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CHANGES IN HEPATIC STRUCTURE IN WILSON'S DISEASE

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The morphologic characteristics of the cirrhosis in Wilson's disease have not been clearly established from the scattered reports in the literature frequently based on single cases. From the time of the earliest reports, the majority of observers have described a coarse, nodular cirrhosis that is now conventionally designated as postnecrotic cirrhosis. Others have described a fine granular liver, now usually called portal, Laennec's, or diffuse septal cirrhosis. Most of the histologic descriptions are indeed sketchy by present standards. The present investigation of a series of cases of Wilson's disease was carried out in an attempt to gain an understanding of the pathogenesis of the cirrhosis in this disorder. Since it seems to be one of the few types of cirrhosis in which at least one etiologic factor, namely, a disturbance of copper metabolism, is established,[1] an investigation of the histologic pattern in fully developed cases or in presumed precursor stages assumes added importance.

MATERIAL AND METHOD

Tissue fixed in formalin or Zenker's solution, paraffin blocks, or prepared sections were obtained with the co-operation of pathologists and clinicians from various areas in the United States. Where possible, in addition to hematoxylin and eosin stained preparations, the following special methods were utilized: the periodic acid-Schiff reaction before and after removal of glycogen with diastase, the Gomori silver impregnation for reticulin, chromotrope-aniline blue, Prussian blue reaction for iron, and Sudan black and oil red O for neutral lipids in frozen sections.

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Most of the patients were in the younger age group and exhibited, except where specifically stated, the characteristic neurologic and biochemical manifestations of Wilson's disease.

Observations

The specimens examined consisted of 19 cases with cirrhosis and 2 without. The sections with cirrhosis exhibited variations in the size of the hepatic nodules, ranging from small aggregates of few cells about 300 μ in diameter to nodules with a maximum diameter of 3 cm. In the smaller nodules, as a rule, the cell plates converged toward the center of the nodule, whereas in the larger nodules a lobular architecture was apparent, with persistent portal tracts and central veins. Occasionally, as many as 4 of each were visible on cross section in one nodule. The central veins appeared more abundant than in the normal liver, and some were found at the periphery of the nodule near the connective tissue septum. In most of the fatal cases, the liver cells near the central veins were necrotic. The degree of degeneration and regeneration of liver cells varied, depending upon the circumstance. The portal tracts occasionally appeared to be connected by perilobular fibrosis. The connective tissue septums between the nodules exhibited considerable variation in width and extent. Veins were abundant in all of them. In the wider septums, forming broad connective tissue bands, "ghost lobules" were apparent following loss of the liver cell plates; in these areas central and portal canals were closely approximated. The former central and intermediate zones consisted of a collapsed framework with only a few cells of mesenchymal character remaining. The peripheral zone was marked by extensively proliferated ductules. The original border of the portal tract, when stained with connective tissue stains, was almost always distinguishable from the collapsed framework since the heavy collagen fibers of the former contrasted well with the wavy membranes in the collapsed parenchyma. The bile ducts and the blood vessels failed to reveal significant alterations. These basic features were modified from case to case, depending upon the activity of the lesion (Table I). In the material examined, several stages could be recognized.

Arrested Stage (4 Necropsies, 1 Biopsy Specimen)

The liver cells revealed little abnormality except for focal necrosis. The cell plates were more than one cell thick in only a few of the smaller nodules. The parenchymal nuclei had the normal vesicular appearance. In the biopsy specimen the cytoplasmic glycogen content appeared average, judging from its vacuolation and granulation. The border between the nodules and the septums was sharp, and the limiting plate was

TABLE I

HISTOLOGIC FEATURES IN VARIOUS STAGES OF CIRRHOSIS IN WILSON'S DISEASE

	Arrested	Partially arrested	Active	Acute parenchymal breakdown	Pre-cirrhotic	Sibling of patient with Wilson's disease
Number of cases	5	6	7	1	1	1
Multilobulated lobules	+	+	+	+	o	o
Broad bands of collapse	o	+	+	+	o	o
Collapse with ghost lobules	o	+	+	+	o	o
Thin connective tissue septums	+	o	o	o	o	o
Conspicuous hepatocellular regeneration	o	+	+	+	o	o
Conspicuous ductular cell proliferation	o	o	+	+	o	o
Defect in limiting plate	o	+	+	+	o	o
Fatty metamorphosis						
Large droplets	o	+	+	+	+	o
Small droplets	o	+	+	+	o	o
Large Kupffer cells	o	+	+	+	o	o
Giant nuclei with glycogen	o	o	+	+	o	o
Moderate nuclei with glycogen	o	+	o	o	o	+
Few nuclei with glycogen	+	o	o	o	+	o
Necrotic liver cells	o	+	+	+	o	o
Nonspecific necrosis	o	+	o	o	o	o
Bile pigment in liver cells	o	o	+	+	o	o
Iron pigment in liver cells	o	o	o	+	o	o
Heavy inflammatory reaction in portal tract	o	o	o	o	o	o

well defined (Fig. 1). The portal tracts showed little inflammatory reaction.

Partially Arrested Stage (6 Necropsies)

Most of the nodules were similar to those described in the previous stage except that many of the peripherally located liver cell nuclei were markedly distended and enlarged up to 20 μ. The nuclear border appeared sharp and refractile. The central portion was unstained, and the periphery of the nuclear droplet gave a positive reaction for glycogen. The nucleolus was displaced to the periphery of the nucleus. In addition, particularly in the smaller nodules, large regenerating liver cells with giant nuclei were common. The hepatic cells were usually arranged in several rows. This was associated with blurred nodular margins and an absence of a limiting plate (Fig. 2). Small numbers of hepatic cells with pigmented and vacuolated cytoplasm lay isolated in the surrounding connective tissue; others appeared in continuity with proliferating ductules. The latter were surrounded by a mononuclear inflammatory exudate containing some neutrophils. In some instances, usually in those with lesser degrees of nuclear degeneration, the disintegrating liver cells exhibited conspicuous fatty metamorphosis. The fat appeared in the form of both large and small droplets.

Active Stage (7 Necropsies)

In all instances except one, large nuclei containing glycogen were prominent features, and in 5 cases these were abundant. The largest of the nuclei measured more than 35 μ in diameter (Figs. 3 and 4). In all but one case, fatty metamorphosis was frequently in the form of small droplets more or less crowding the cytoplasm but not displacing the nucleus (Fig. 7). In some instances, large, single fat droplets filled the entire cell and pushed the nucleus to the side (Fig. 8). In 2 cases almost all liver cells in all nodules exhibited severe fatty metamorphosis. Disintegration of the fatty cells was characterized by acidophilic coagulation and bile pigmentation as well as by the accumulation of nonglycogenic PAS-positive intracytoplasmic granules. Neighboring Kupffer cells were conspicuously enlarged and contained cellular breakdown products, some of which were PAS-positive. Fat droplets up to 6 μ in diameter and amorphous acidophilic material also appeared within Kupffer cells (Fig. 9). The fatty metamorphosis and disintegration of liver cells, as well as the Kupffer cell reaction, were particularly marked in smaller nodules. The parenchyma bordering upon the connective tissue was not sharply outlined. Regeneration was prominent and characterized by giant liver cells with large nuclei. In sections impregnated

with silver, recent collapse of the pre-existing reticulin framework was noted. Ductular proliferation and inflammatory reaction were generally prominent, and plasma cells were, in some cases, frequently gathered about the proliferated ductules. Bile stasis with plugs in bile ductules was found in 4 cases.

Acute Parenchymal Breakdown (One Necropsy)

While glycogen degeneration of the nuclei and fatty metamorphosis were prominent but less widespread than in other cases, necrosis of liver cells was reflected by conspicuous brown cytoplasmic pigmentation and PAS-positive granulation. Neighboring Kupffer cells were heavily loaded with bile pigment and PAS-positive material. Approximation of the reticulin framework, indicating recent collapse, was noted in many areas (Fig. 6). The collapsing zone appeared to merge with the neighboring connective tissue septums (Fig. 5). They contained irregularly proliferating ductules, many scavenger cells and isolated bile pigment-laden liver cells.

Precirrhotic Fatty Metamorphosis (One Biopsy Specimen)

The liver of a patient with the clinical and biochemical features of Wilson's disease showed patchy areas of fatty metamorphosis mainly in the form of large droplets without specific lobular distribution (Fig. 10). There was practically no liver cell necrosis, and the Kupffer cells contained only scant amounts of PAS-positive material in diastase-treated sections. Very few of the liver cell nuclei exhibited glycogen change. Small amounts of copper were demonstrated in the parenchymal cells, using the technique of Uzman.[2]

Sibling of a Patient with Wilson's Disease (Two Biopsy Specimens)

The surgical biopsy specimens were obtained from one child at the ages of 3½ and 4½ years. The sister of this patient had evidence of fully developed hepatolenticular degeneration. Ceruloplasmin had been absent from the serum of the patient since birth, but no other clinical or laboratory evidence of hepatic or neurologic dysfunction was detectable.[3] Both specimens exhibited normal hepatic structure except for the presence of enlarged glycogen nuclei in the periphery of the lobules (Fig. 11).

Control Group

For comparison, 15 examples of active postnecrotic cirrhosis not associated with Wilson's disease were gathered from the necropsy files of the Department of Pathology, Mount Sinai Hospital. Glycogen-contain-

ing nuclei were observed in 3 instances. In one this feature was fairly prominent though not as extensive as in most of the cases of active Wilson's disease. The nuclei in these cases did not attain the unusual size observed in the active stages of Wilson's disease. Fatty metamorphosis of some degree was noted in 8 of the control cases. Necrosis and breakdown of fatty cells was noted in only one instance, and this occurred without enlarged Kupffer cells.

Discussion

In all necropsy specimens from cases of hepatolenticular degeneration, the typical lesion of postnecrotic cirrhosis was found. This was characterized by 3 cardinal manifestations[4,5]: (a) irregular distribution of the postnecrotic alterations as reflected by preserved lobular architecture in many nodules; (b) stromal collapse with the formation of septums of varying width; (c) conspicuous parenchymal regeneration. The existence of postnecrotic cirrhosis as a regular feature of Wilson's disease is confirmed by those cases reported in the literature, in which either the illustrations or the histologic descriptions provide adequate information as to the character of the cirrhosis.[6-10]

Postnecrotic cirrhosis may result, at least in some instances, from viral hepatitis. Several factors are incompatible with the assumption that the cirrhosis of Wilson's disease might be the sequel of intercurrent viral hepatitis. Some of the manifestations of acute viral hepatitis seen in postnecrotic cirrhosis, i.e., acidophilic bodies and single cell necrosis, with accumulation of mononuclear cells in place of the liver cells that have disappeared,[11,12] were not observed in Wilson's disease. There were two features which were conspicuous, however, in Wilson's disease. These were not evident as prominently or as frequently in examples of postnecrotic cirrhosis, particularly those following viral hepatitis. One was the presence of both large and small fat droplets in hepatic cells. This is occasionally found in postnecrotic cirrhosis, presumably as the result of complicating nutritional disturbance. In Wilson's disease, however, fatty metamorphosis is a regular feature and apparently is associated with the activity of the process, particularly in relation to foci of degenerating liver cells. The appearance of large Kupffer cells in such areas is also noteworthy and would seem to indicate an irritation of these mesenchymal cells by fat. This is not common in other types of cirrhosis. The fatty metamorphosis seemed to precede the cirrhotic alterations. This was suggested by one of our biopsy specimens and has been indicated by other recorded observations.[9,13]

A second important feature is glycogen degeneration of nuclei, which appears to be outstanding in the active stages of the cirrhosis in Wilson's

disease. Accumulation of glycogen within hepatic nuclei has been observed with significant frequency in diabetes.[14] It has been recorded as an incidental occurrence in other conditions and has been produced experimentally by the *in vivo* perfusion of rat liver with hypotonic saline.[15] The physiologic implications of this phenomenon are not wholly clear. It, too, occurs in other types of postnecrotic cirrhosis but not with the frequency and regularity that is manifested in Wilson's disease. In the majority of our cases the nuclear change appeared in many more cells than was usually the case in the livers of patients with diabetes. Even more remarkable was the large size of the glycogen-containing nuclei, particularly in the active stages of the cirrhosis. Possibly the glycogen degeneration is, in some way, related to the underlying metabolic abnormality in this condition. This is suggested by the occurrence of this alteration in the liver of a clinically normal sibling of a patient with Wilson's disease. This child exhibited no abnormality except for the absence of serum ceruloplasmin. To our knowledge, glycogen degeneration of hepatic nuclei has not been specifically emphasized in previously reported cases of Wilson's disease. However, nuclear vacuolation has been mentioned by several authors,[8,16,17] and in at least one report[7] it was ascribed to the presence of intranuclear glycogen. The vacuolation can be recognized in the illustrations in several reports.[17-19] The possibility that the nuclear vacuolation is an incidental feature attributable to the parenteral administration of supportive fluids (intravenous glucose or saline infusions) must also be entertained. This would apply particularly in the case of patients receiving supportive care in the terminal stages of their illnesses.

Although the relation of fatty metamorphosis and nuclear glycogen degeneration to the metabolic disturbance in Wilson's disease is not clear, these alterations appear to be associated with the breakdown of hepatic parenchyma that leads to the postnecrotic cirrhosis in Wilson's disease.

The specimens investigated reveal various features in the cirrhosis of Wilson's disease, indicating a range from arrested to active stages. In addition to the other recognized criteria of activity in postnecrotic cirrhosis, fatty metamorphosis and glycogen degeneration of the liver nuclei occur in Wilson's disease. In one case in which massive necrosis and beginning collapse reflected the active development of characteristic features of postnecrotic cirrhosis, the most actively degenerating cells appeared bile-laden. It is likely, therefore, that in the massive necrosis underlying postnecrotic cirrhosis, other processes are involved as well. The observations cited indicate that the cirrhosis seen in Wilson's disease may develop through stages that differ from those observed following viral hepatitis.

Summary

Histologic observations of the liver in 20 examples of hepatolenticular degeneration (Wilson's disease) indicate that the cirrhosis associated with this condition is postnecrotic in type, with stages varying from those which are completely arrested to others which are very active. Features not found, at least with significant regularity or extent, in other types of postnecrotic cirrhosis are degeneration of fat-containing liver cells, the appearance of excessively large Kupffer cells, and glycogen degeneration of the liver cell nuclei. The fatty metamorphosis and nuclear alterations apparently precede the development of cirrhosis and appear to contribute at least in part, to the necrosis and stromal collapse characteristic of postnecrotic cirrhosis.

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[*Illustrations follow*]

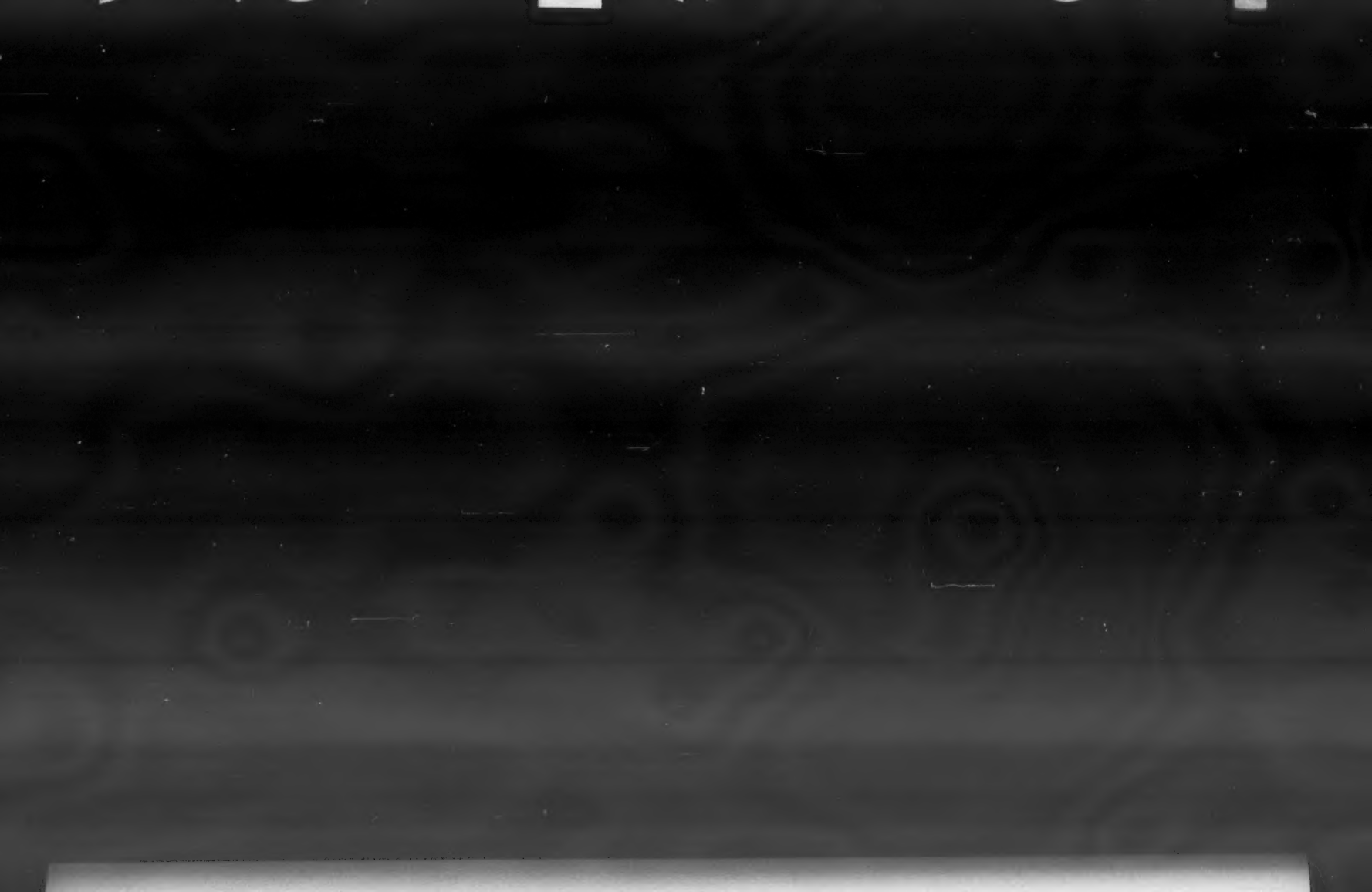
Legends for Figures

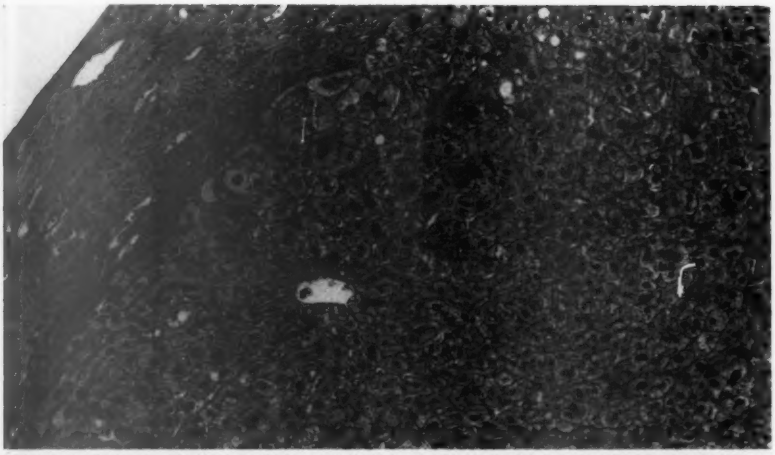
Except where indicated, the photographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. Arrested stage of postnecrotic cirrhosis in Wilson's disease. The limiting plate of the nodule is well defined, and the inflammatory reaction in the broad septum is subdued. × 63.

Fig. 2. Partially arrested stage. The margins of the nodules are not sharp. Hepatic cell nuclei distended with glycogen are present at the periphery of the nodules (arrow) or near portal tracts. × 63.

Fig. 3. An active stage with destruction of the limiting plate producing ragged nodule borders. Many liver cell nuclei are vacuolated and contain glycogen. The septum exhibits a diffuse inflammatory reaction and ductular proliferation. × 63.





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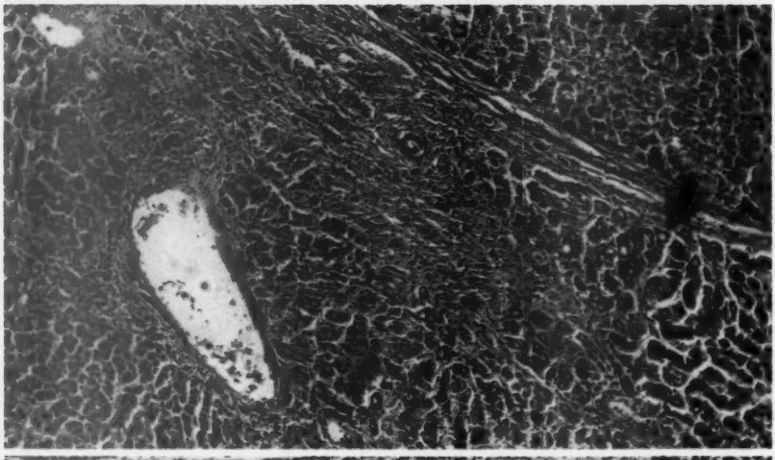

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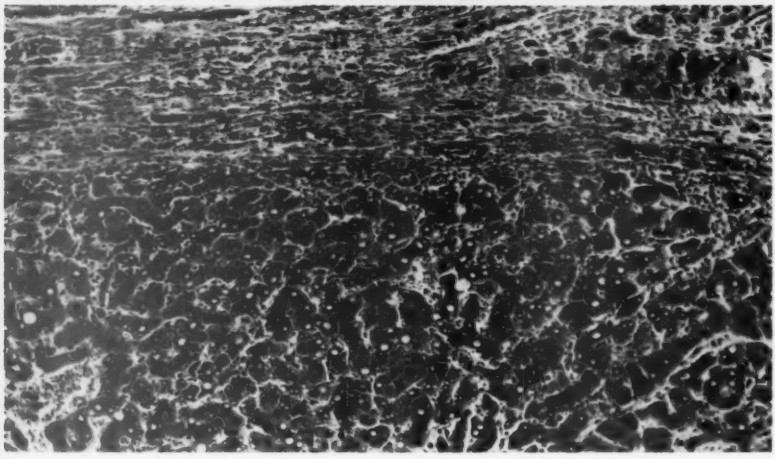

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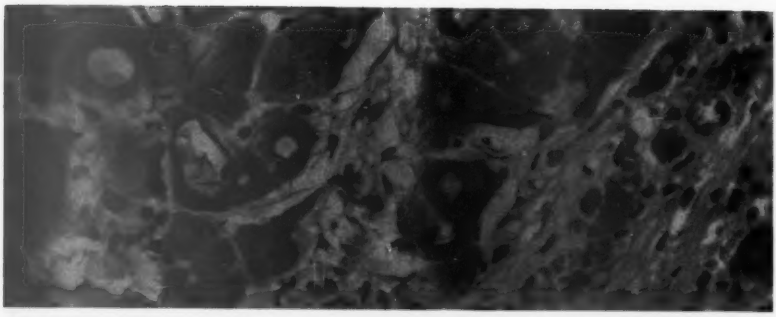
FIG. 4. Nuclear glycogen degeneration in an active stage of cirrhosis in Wilson's disease. The nuclei are extremely enlarged and ballooned; nucleoli are displaced to the periphery. × 400.

FIG. 5. Stage of acute parenchymal breakdown. Necrosis is widespread, and the collapsing hepatic parenchyma appears to merge with adjacent septums. Fatty metamorphosis (straight arrow) and glycogen degeneration of nuclei (curved arrow) are noted. × 120.

FIG. 6. Acute parenchymal breakdown. The reticulin framework is closely approximated (arrow). Gomori reticulin stain. × 120.



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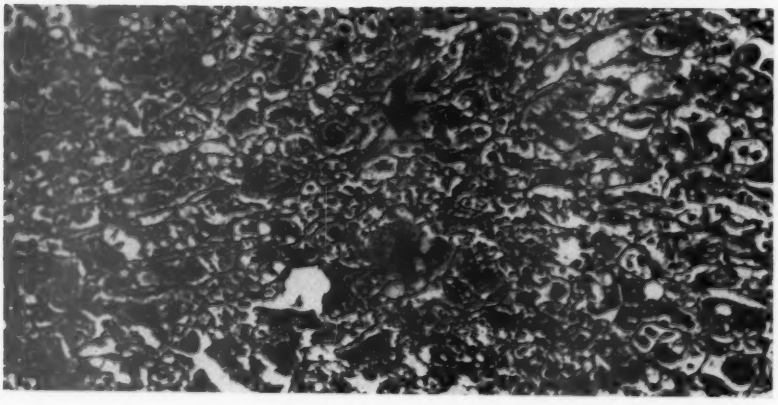

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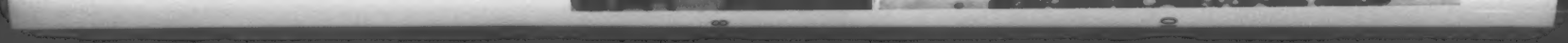
FIG. 7. Active stage. Fatty metamorphosis and nuclear glycogen degeneration is typically seen at this stage. × 120.

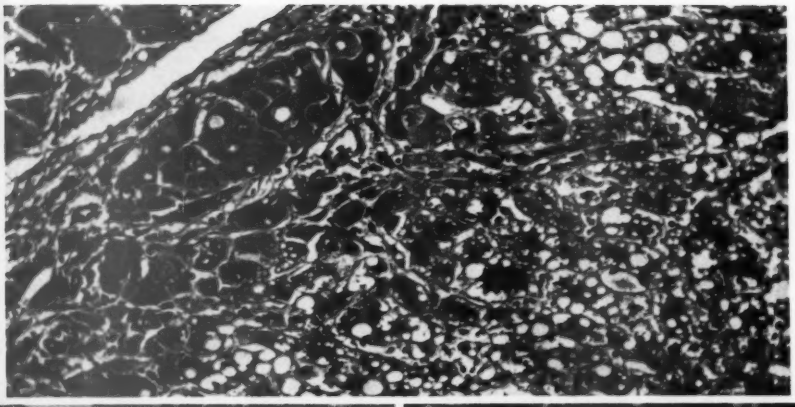
FIG. 8. Active stage. Large fat droplets appear within the cytoplasm of parenchymal cells, and a large Kupffer cell has a granular cytoplasm (arrow). × 240.

FIG. 9. Active stage. Enlarged Kupffer cells with vacuolated cytoplasm are associated with fatty metamorphosis and pigment deposition in liver cells. × 240.

FIG. 10. Precirrhotic stage. Liver biopsy from a patient with the clinical and biochemical features of Wilson's disease. Only extensive fatty metamorphosis is evident. × 63.

FIG. 11. Liver biopsy from an asymptomatic sibling of a patient with Wilson's disease. The sibling showed only absence of serum ceruloplasmin. Large nuclei containing glycogen are present in some of the hepatic cells at the periphery of lobules. × 120.





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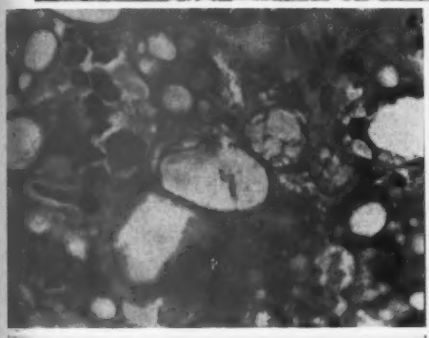

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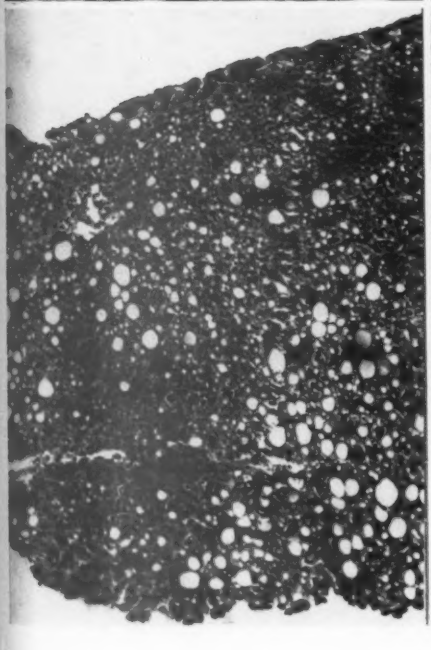

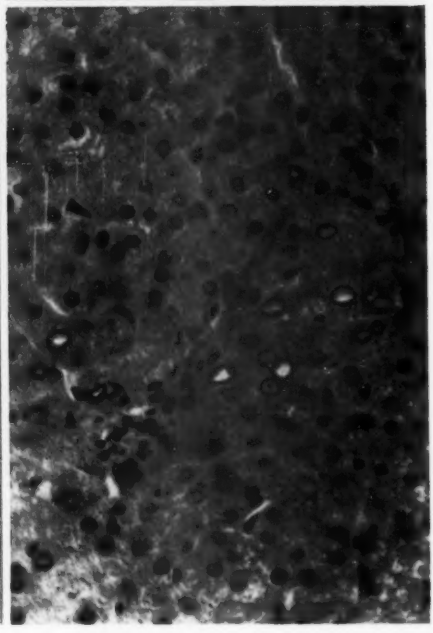

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EXPERIMENTAL PIGMENT CIRRHOSIS

Its Production in Rats by Feeding a Choline-Deficient Diet With Excess Iron

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Attempts to reproduce hemochromatosis in animals experimentally have had only limited success to date. By administering iron in various forms, injecting toxic substances, or feeding diets which promote excessive absorption of dietary iron, conditions have been achieved in which there have been large quantities of iron in many tissues of the body. Pigment cirrhosis, an essential lesion of hemochromatosis, however, has not resulted consistently. In the present experiment, hepatic cirrhosis analogous to pigment cirrhosis in humans, together with excess iron in most organs of the body, was produced in rats by feeding a choline-deficient diet with added iron, followed by correction of the deficient diet. This experiment was undertaken because the observations in a study of human subjects with hemochromatosis suggested that factors related to nutritional deficiency might have caused both cirrhosis and excess iron absorption.[1] In brief, these findings were: (a) an unusually frequent occurrence of hemochromatosis in a hospital where a type of cirrhosis related to alcoholism and malnutrition was also very common; (b) the frequent observation of residual fat and "alcoholic hyalin" in the livers of patients with hemochromatosis, suggesting that there had earlier been a nutritional or alcoholic type of cirrhosis; (c) a frequent history of alcoholism in patients with hemochromatosis, many patients stating that they had not drunk alcohol in recent years (such recent abstinence might have obscured a history of past malnutrition in additional patients; correction of dietary deficiency would have resulted in the mobilization of fat from the liver, leaving chiefly a portal type of cirrhosis with siderosis); (d) in an analysis of patients with hemochromatosis, hemosiderosis, and an intermediate group with what was termed advanced hemosiderosis, no definitive clinical or pathologic difference was found among the 3 groups, suggesting that they were gradations of a single disease.

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Review

Attempts to produce hemochromatosis experimentally might be reviewed in 3 categories: (a) administration of iron and related substances; (b) administration of toxic substances such as copper, ethionine, and alloxan; and (c) feeding deficient diets which cause excess absorption of dietary iron.

The earliest important work in the first group, in which citrated blood was given intravenously to rabbits for a 6-month period, was that of Rous and Oliver in 1918.[2] After a few weeks they found hemosiderin in the spleen and bone marrow. Later the liver and kidneys were affected, and after 6 months there was extensive pigmentation of other organs, including the spleen, pancreas, heart, adrenals, skin, and mammary glands. Iron was not present in the stomach, lungs, thyroid and brain. Although the authors considered that they had produced hemochromatosis, they did not find cirrhosis of the liver. Polson,[3-5] in experiments extending for as long as 4 years, gave colloidal iron to rabbits intravenously, intramuscularly, and intraperitoneally, in doses of 0.1 to 0.3 mg. per kg. of body weight. He was unable to produce either cirrhosis of the liver or iron deposits in pancreatic epithelium, although he found extensive iron deposits in the liver, spleen, kidneys, lungs, bone marrow, and adrenals. Cappell[6] gave saccharated iron oxide to rats and mice intravenously for as long as 14 months, and achieved approximately the same results as Polson. Following this, many workers studied the effects of injecting various forms of iron and red blood cells into rabbits, mice, rats, guinea pigs, and dogs, but fibrosis of the liver did not result.[7-11] The work of Finch and colleagues[12] deserves particular mention. This group gave intravenous iron ascorbate and saccharated iron oxide to dogs in a dose of 2 mg. per kg. for as long as 2 years and in a few animals produced hepatomegaly, ascites and evidence of liver failure. There was slight hepatic fibrosis. After excess iron was present in the tissues, the dogs were bled by phlebotomy, and it was found that not all of the tissue iron was mobilized and utilized for hemoglobin synthesis. In addition, it was noted that the ratio and behavior of ferritin to hemosiderin in the tissues of dogs containing excess iron was the same as in a patient with hemochromatosis.

Several attempts have been made to reproduce hemochromatosis by administering toxic substances. Perhaps the most controversial of these was the work of Mallory, Parker, and Nye, who from 1920 on ascribed hemochromatosis to chronic poisoning with copper.[13-16] In 1898 Baum and Seeliger[17] had fed copper to animals and produced focal necrosis of liver cells, accompanied by hemosiderin deposits, but they had not

been interested in the relation to hemochromatosis. In 1906 Kobert,[18] writing of toxic substances, introduced a concept, later used by Mallory, that adding a dilute copper solution to hemoglobin resulted in a substance which he termed "cuprohemol." Mallory and co-workers fed 23 rabbits and one monkey copper in doses of 0.1 to 0.2 gm. per day for as long as one year, and found fibrosis of the liver in 3 animals. There was hemofuscin and hemosiderin pigment in several of the animals. From this and from later work it was postulated that hemochromatosis was caused by chronic poisoning with copper, which caused hemolysis of red blood cells and deposition of "cuprohemol" in the liver. This substance was considered to be similar to or identical with hemofuscin. Hemofuscin was believed to contain, in addition to copper, iron in a masked form which did not stain by the usual iron methods. With the passage of time, it was postulated that hemofuscin was transformed into hemosiderin. This view was based on two additional concepts. One was that of von Recklinghausen, who believed the characteristic features of hemochromatosis to be the presence of hemosiderin and hemofuscin pigments, and who did not emphasize the constancy of cirrhosis.[19] The second was that of Aschoff and Hess, who in 1904 expressed the belief that in hemochromatosis, hemofuscin pigment was first deposited in tissues and was later transformed into hemosiderin.[20] The work of Mallory was criticized by Sheldon[21] on the grounds that he did not have controls, and that the same proportion of "normal" rabbits in his study developed spontaneous fibrosis of the liver, perhaps as a result of endemic infection.[18-22] Moreover, other workers could not duplicate his results,[23-25] although some claimed to do so.[26] It has also been pointed out that hemofuscin was found in the livers of normal rabbits maintained on diets containing carrots and turnips.[22,23]

Additional investigations of the effects of toxins have been those utilizing ethionine and alloxan. Kinney, Kaufman and Klavins[27] gave the methionine antagonist DL-ethionine to rats and mice, and found that in addition to ethionine lesions in the liver, pancreas, and testes, there was increased iron absorption from a normal diet. When 2 per cent ferric citrate was added, even more iron was found in tissues, and the lesions bore a superficial resemblance to those of hemochromatosis. Herbut, Watson and Perkins[28] produced alloxan necrosis of the liver and pancreas in rabbits and observed slight hemosiderin deposits in the liver and spleen. Neither of the latter two experiments produced lesions closely analogous to those of human hemochromatosis.

Pancreatectomy as a means of inducing hemosiderosis was first reported by Taylor, Stiven, and Reid in 1931.[29] In studying the effects of pancreatectomy in cats, they noted that in one animal which survived for

224 days there was considerable hemosiderosis unaccompanied by cirrhosis of the liver. In further work they found that after either extirpation of the pancreas or ligation of the pancreatic ducts, iron deposits occurred in the liver, spleen, pancreas, lymph nodes, stomach, small intestine, and adrenals; this was accentuated if an iron-rich diet was fed.[30] Similar experiments by Gillman, Gillman, Mandelstam and Gilbert[31] in cats, and by Kinney and associates in dogs[32] confirmed the observation of widespread hemosiderosis without cirrhosis. The manner in which pancreatic duct ligation causes excess iron absorption is not known; choline deficiency may be implicated. Best, Hershey and Huntsman[33] were led to the discovery of choline when they noted that pancreatectomized dogs maintained on insulin developed fatty liver and died; deficiency of choline was eventually found to be responsible. As will be discussed below, choline-deficient diets cause abnormal iron absorption.

Pyridoxine deficiency, which in experimental animals produces dermatitis, demyelinization of peripheral nerves, microcytic anemia, elevated serum iron, fatty liver, and other lesions, leads to excess iron and copper absorption from the diet, with hemosiderosis of the liver, spleen, and bone marrow. Cirrhosis of the liver does not occur.[34-37] Because hemosiderosis can be produced by feeding a deficient diet of corn grits, it is of interest that corn meal extracted with alcohol was the first pyridoxine-deficient diet to be used.[38]

Some of the most significant investigations into the problem of iron absorption and metabolism have been carried out by Kinney, Hegsted and Finch.[39-41] This group reported that deficient diets consisting chiefly of corn grits led to excess iron absorption with hemosiderosis of the liver, spleen, bone marrow, duodenum, kidney, adrenals and testes, although no iron was found in the brain, lung, heart, pancreas, thyroid or parathyroid. This work has been confirmed and extended,[42-44] and further investigation has shown that excess iron in a normal diet leads to deposits in the liver, spleen, bone marrow, and in the connective tissue of the pancreas, heart, stomach and intestine. The explanation for excess iron absorption on a corn grits diet is not yet clear; addition of phosphate to the diet up to certain levels gives partial protection, and amino acid imbalance has been implicated. A further example of dietary hemosiderosis is the study of Wissler, Bethard, Barker and Mori[45] who produced hemosiderosis in hamsters by adding polyoxyethylene sorbitan monolaurate (Tween 20) to the diet. The effect on iron absorption of feeding a choline-deficient diet has also been previously investigated. Kaufman and co-workers appear to have been the first to attempt to produce hemochromatosis in this way.[46] Rats fed a low-protein, low-choline diet combined with excess iron or copper or both, did not exhibit

lesions resembling hemochromatosis after 8 months; there was, however, increased iron in the liver, spleen, and kidneys. Seki and Chalmers [47] also fed an 8 per cent protein, low choline diet to rats in studies carried out to determine the effects of dietary liver disease on iron absorption and metabolism.

Material and Methods

In this investigation two experiments were performed. Experiment I was an attempt to learn whether a diet that caused fatty liver and cirrhosis in rats would also cause excess absorption of iron from the gastrointestinal tract, and whether, after cirrhosis was induced, removal of the deficient diet would lead to a histologic lesion analogous to that in human pigment cirrhosis. In addition, hepatic cell regeneration as a consequence of increased deposits of hepatic iron was studied. This was investigated by injecting tritiated thymidine before animals were sacrificed, and quantitating in autoradiographs the number of hepatic cell nuclei that utilized the labeled thymidine for deoxyribonucleic acid (DNA) synthesis.[48,49] Experiment II was undertaken to learn whether feeding a choline-deficient diet caused alterations in the gastrointestinal tract, liver, or other organs, so that increased absorption of iron continued after the deficient diet was removed.

Experiment I

Fifty-six male rats of the Sprague-Dawley strain (purchased from the Charles River Breeding Laboratories, Cambridge, Massachusetts), of the same age and separated by litter mates, were used. The experiment was begun when the animals were 82 days old, at which time they weighed 200 to 300 gm. Animals were housed in individual suspended wire mesh cages in a constant temperature, constant humidity room. All feeding was *ad libitum;* body weights were determined weekly. Sixteen animals were separated into 2 groups, 8 per group; one group was fed a choline-deficient diet [50] with 3 per cent powdered ferric ammonium citrate, N.F. brown powder (Mallinckrodt Company, St. Louis, Missouri), which contained 17 per cent iron. The second group was fed Purina Laboratory Meal with 3 per cent added iron. After 21 days on the diets, all animals were sacrificed by ether anesthesia, and the livers were perfused with saline. Portions of liver were fixed in 10 per cent neutral buffered formalin [51a]; these were stained for iron by the ammonium sulfide [51b] and the potassium ferrocyanide [51c] methods. The remaining liver was used to determine the iron content chemically.[52-54]

The 40 remaining rats were fed the following diets: Seven rats were fed Purina Laboratory Meal with 3 per cent iron; 4 control rats were fed

the powdered laboratory meal without added iron. Seven rats were fed the choline-deficient diet with 3 per cent iron; 4 control rats were fed the choline-deficient diet alone. Five rats were fed the choline-deficient diet to which 1 per cent choline chloride and 3 per cent iron were added; 7 rats were given the choline-deficient diet with 1 per cent choline chloride but without added iron. Six rats were fed the choline-deficient diet with 3 per cent iron, and after 69 and 97 days of this diet, they were fed Purina Laboratory Chow without added iron until sacrificed. After animals had been on the diets for 69, 97, 137, or 156 days, one or more animals from each group, with the exception of those on the choline-deficient diet that were restored to a normal diet, were sacrificed by decapitation. Four hours prior to sacrifice a single intraperitoneal injection of tritiated thymidine (Schwarz Laboratories, Inc.; specific activity 0.360 μc per mM) was given to each animal in a dose of 0.7 μc per gm. of body weight. At the time of sacrifice a sample of blood was obtained for hematocrit determination.[55] Tissues were fixed in 10 per cent neutral buffered formalin and in Zenker's solution with 5 per cent glacial acetic acid. Histologic sections were stained with hematoxylin and eosin,[51d] phloxine methylene blue,[51e] van Gieson's stain for connective tissue,[51f] oil red O fat stain in paraffin sections for ceroid,[56a] acid-fast stain,[56b] benzidine stain for hemoglobin,[57] Mallory's basic fuchsin stain for hemofuscin,[58] and ammonium sulfide and potassium ferrocyanide stains for iron. Nearly all tissues of the body were examined. For preparation of autoradiographs, stripping film (Film AR 10, Kodak Ltd., London) was placed over unstained paraffin sections cut at 6 μ. They were then exposed at 4° C. for 4 weeks, developed in Kodak D-19 developer for 7 minutes, passed through distilled water, fixed in Kodak Acid Fixer for 10 minutes, washed, stained with freshly prepared hematoxylin and mounted in polyvinylpyrrolidone (PVP).[59] Tritium-labeled hepatic cell nuclei were quantitated in 100 consecutive fields at a magnification of 400 times, and the results were expressed per 100,000 hepatic nuclei. Mitotic figures in hepatic cells were quantitated in 50 consecutive fields at the same magnification in histologic sections, and the results were similarly expressed. If no mitotic figures were seen in 50 fields, the results were expressed as zero.

Composition of the Purina Laboratory Meal, according to the manufacturer's specifications, was as follows: crude protein, not less than 23 per cent; crude fat, not less than 5 per cent; crude fiber, not more than 6 per cent; nitrogen-free extract, not less than 44 per cent; ash, not more than 9 per cent. Ingredients were: meat meal, dried skimmed milk, wheat germ, fish meal, liver meal, dried beet pulp, corn grits, oat middlings, soybean oil meal, dehydrated alfalfa meal, feeding cane molasses, vitamin

B_{12} supplement, riboflavin supplement, brewers' dried yeast, thiamine, niacin, vitamin A feeding oil, vitamin D activated plant sterol, 0.5 per cent defluorinated phosphate, 0.5 per cent iodized salt, 0.02 per cent manganese sulphate.

Composition of the choline-deficient diet was as follows: peanut meal (Dothan Oil Mill Company, Dothan, Alabama), 12 per cent; gelatin, 6 per cent; vitamin-free casein, 3 per cent; fibrin, 1 per cent; beef fat, 20 per cent; corn oil, 2 per cent; cod liver oil concentrate, 0.015 per cent; cystine, 0.5 per cent; cellulose flour, 2 per cent; sucrose, 47.5 per cent; salts, 5 per cent; and vitamin powder, 1 per cent.

Salts per 100 gm. contained: calcium lactate, 35 gm.; calcium carbonate, 5.06 gm.; $Ca(H_2PO_4) \cdot H_2O$, 14.60 gm.; K_2HPO_4, 6.46 gm.; $NaH_2PO \cdot H_2O$, 18.76 gm.; NaCl, 9.34 gm.; $MgSO_4$ (anhydrous), 7.18 gm.; $ZnSO_4 \cdot 7\,H_2O$, 0.035 gm.; $CuSO_4 \cdot 3\,H_2O$, 0.039 gm.; KI, 0.00039 gm.; Fe citrate, 3.2 gm.; $MnSO_4 \cdot 4\,H_2O$, 0.33 gm.

Vitamin powder per 1,000 gm. contained: thiamine, 500 mg.; riboflavin, 250 mg.; pyridoxine, 200 mg.; calcium pantothenate, 1 gm.; nicotinic acid, 1 gm.; vitamin-free casein, 997.06 gm. Alpha tocopherol acetate was added to the diet to provide each rat with one drop per week.

TABLE I

IRON CONTENT OF THE LIVER IN RATS FED A CHOLINE-DEFICIENT DIET WITH 3 PER CENT IRON FOR 21 DAYS

	No. of rats	Average wt. of liver at sacrifice (gm.)	Hepatic nonhemin iron*		
			μg. per gm. of liver	Mg. per 100 gm. of liver	Total hepatic iron (mg.)
Group I:					
Choline-deficient diet with 3% iron for 21 days	8	27.4 ± 4.2†	215.5 ± 93.0	21.5 ± 9.3	6.0 ± 2.8
Group II:					
Laboratory meal diet with 3% iron for 21 days	8	15.7 ± 2.2	114.4 ± 49.9	11.4 ± 4.9	1.8 ± 0.9

* Iron content was determined in 0.5 gm. of liver.
† Standard deviation.

Experiment II

In this experiment 16 male rats, paired by litter mates, were used. At the age of 99 days, 8 rats were fed the choline-deficient diet, and 8 control rats were fed Purina Laboratory Chow for 85 days. Then for an additional 21 days, both groups were fed Purina Laboratory Chow in order to correct the effects of the deficient diet. A small surgical biopsy

of the liver, of approximately 0.2 gm., was obtained from each animal; this was frozen and stored for later iron determination. All animals were then fed Purina Laboratory meal with 3 per cent ferric ammonium citrate, N.F. brown powder, for 40 days. At the end of this time the animals were sacrificed by ether anesthesia, and the livers were perfused with saline, and weighed; small portions were fixed in 10 per cent neutral buffered formalin, and the remainder was frozen and stored. Histologic sections of liver and other organs were stained as in Experiment I. The frozen portions of liver were used to determine the amount of tissue iron present.

Results

Experiment I

Rats fed the choline-deficient diet with added iron absorbed and deposited more iron in the liver than did rats fed a diet of Purina Laboratory Meal with added iron (Table I). The differences were significant by the *t* test beyond the 0.05 level. The average gain in body weight during the 21-day period was 5.6 per cent for rats fed the choline-deficient diet with iron and 2.1 per cent for rats fed laboratory meal with iron.

Choline-deficient Diet Alone. In rats fed the choline-deficient diet without added iron, fatty liver and cirrhosis developed. Small amounts of histologically stainable iron were demonstrable in macrophages and in fibrous tissue at 156 days, but none was found in hepatic cells, and there was no resemblance to hemochromatosis. No iron was demonstrable in other organs, and no pancreatic fibrosis or inflammation was found; as in previous studies with experimental cirrhosis,[60] it was found that rats did not develop pancreatic lesions. Ceroid [61] was demonstrable in the livers and lungs of animals, once markedly fatty liver developed.

Choline-deficient Diet Plus 3 Per Cent Iron. In rats fed the choline-deficient diet with 3 per cent iron, the same sequence of fatty liver and cirrhosis was observed, but in addition, hemosiderin deposits were found in the liver and other organs from 69 days on. In the liver, iron was demonstrated chiefly in hepatic cells, and its appearance preceded the onset of cirrhosis. Iron was also found in macrophages, fibrous tissue, endothelium, and in connective tissue in the liver; cells in all portions of the liver lobule, including the central vein regions were affected. Ceroid was also present. The interstitial tissue of the pancreas, heart and adrenal showed iron deposits, as did the interstitial and parenchymal cells of the stomach. The proximal tubular epithelium of the kidney, the connective tissue of the villi of the duodenum, and in particular the interstitial tissue of the colonic mucosa, as well as the submucosal and subserosal connective tissue of the entire gastrointestinal tract, showed

iron deposits. The alveolar cells and basal mucosal cells of the bronchi occasionally showed small amounts of iron. The testes exhibited fibrosis, and iron was present early and in relatively large amounts about blood vessels and in interstitial tissue. Abundant iron was found in abdominal lymph nodes and in the pulp tissue of the spleen, sparing the malpighian corpuscles. No iron was demonstrated in the skin, and in only two animals did rare macrophages in the bone marrow contain iron. The features in this group of cases appeared to resemble human cases of fatty portal cirrhosis with hemosiderosis.

Restoration to Normal Diet After Choline-deficient Diet Plus 3 Per Cent Iron. In rats fed the choline-deficient diet with 3 per cent iron, then given a diet of laboratory chow, one of two patterns resulted. In rats that had developed cirrhosis, the lesion, after fat had been mobilized from the liver, was one of portal cirrhosis with heavy deposits of hemosiderin, analogous to pigment cirrhosis in humans (Fig. 1). Small amounts of hemofuscin were found in the connective tissue of portal areas. Hemosiderin was not found as small granules in bile duct epithelium of the rat, as is characteristic of human pigment cirrhosis, but it was present in connective tissue of portal areas adjacent to bile ducts and also in the connective tissue beneath bile duct epithelium. In the livers of two rats a small collection of nonpigmented hepatic cells (Fig. 2) was encountered. This suggested an analogy to pigment cirrhosis in humans, in which nonpigmented hepatic nodules are commonly found.

In other organs, the distribution of iron was similar to that found in animals fed the deficient diet with added iron. Figure 3 illustrates iron deposits in the pancreas in this group. In animals which did not consume the diet avidly and did not develop cirrhosis,[62] the fat was mobilized from the liver when a normal diet was restored. Little or no fibrosis remained, and iron deposits in the liver and other organs were identical to those in rats in the above group. The pattern (Fig. 4) thus resembled that observed in cases of advanced hemosiderosis in humans.[1]

Choline-deficient Diet Plus 1 Per Cent Choline and 3 Per Cent Iron. The deposition of iron in the liver and other tissues of animals fed the choline-deficient diet to which 1 per cent choline and 3 per cent iron were added was similar to the above groups, but the amount of iron appeared to be less. In the livers of this group there were occasional globules of fat in scattered hepatic cells, which may have meant that the added choline was not sufficient to protect against choline deficiency.

Laboratory Meal Plus 3 Per Cent Iron. Rats fed Purina Laboratory Meal with 3 per cent iron, when sacrificed after 69 days on the diet, showed slight iron deposits in Kupffer and hepatic cells. The iron appeared in cells about the portal areas, with almost complete sparing of

TABLE II

EFFECT OF CHOLINE-DEFICIENT DIET AND OF DIETARY IRON ON BODY WEIGHT, HEMATOCRIT, AND HEPATIC CELL REGENERATION OF RATS

Diet							
Type	No. days fed	No. days dietary supplement*	No. rats	Average wt. at sacrifice (gm.)	Average hematocrit per 100 cc.	Average no. of H^3-labeled hepatic nuclei†	Average no. of hepatic mitotic figures‡
Laboratory chow	69	0	1	397	48	65	10
	97	0	1	491	42	228	21
	137	0	1	351	43	280	0
	156	0	1	336	44	126	0
Laboratory chow with 3% iron	69	0	2	343	48	286	63
	97	0	1	315	42	230	0
	137	0	2	279	47	64	0
	156	0	2	253	45	946	0
Choline-deficient	69	0	1	396	47	474	12
	97	0	1	351	41	2,241	114
	137	0	1	310	44	260	0
	156	0	1	340	41	1,063	24
Choline-deficient with 3% iron	69	0	2	306	46	440	51
	97	0	1	190	45	677	22
	137	0	2	314	44	1,037	24
	156	0	2	341	46	1,418	12
Choline-deficient with 3% iron; then laboratory chow	69	26	2	317	42	176	23
	97	42	2	321	41	191	0
	97	88	2	379	45	174	3

* Six rats were fed the choline-deficient diet with 3 per cent iron, and after 69 and 97 days of this diet, they received Purina Laboratory Chow without added iron until sacrificed.

† The number of H^3-labeled hepatic nuclei per 100,000 hepatic nuclei in autoradiographs.

‡ The number of hepatic mitotic figures per 100,000 hepatic nuclei in histologic sections.

those in central vein regions. There were slight iron deposits in the interstitial tissue of the colon, testis, and in the proximal tubular epithelium of the kidney. Iron was found in lymph nodes and in the pulp of the spleen; later, particularly at 156 days, it was found in the liver, in both Kupffer and hepatic cells. To a minimal degree, iron appeared in interstitial tissue of the pancreas, heart, adrenal, testis, lung, and in the pulp of the spleen. In the gastrointestinal tract, heavy deposits were found in interstitial tissue of the duodenum and stomach. None was found in the bone marrow or skin.

Laboratory Meal Without Added Iron. In animals fed laboratory meal without added iron, hemosiderin deposits were found only in the pulp of the spleen. Iron was not present elsewhere, nor was fibrosis of the liver encountered in control animals.

Autoradiographic and Other Studies. Table II summarizes the autoradiographic and other studies in 5 groups of animals. Rats fed 3 per cent iron in addition to laboratory meal weighed slightly less than those fed the meal alone. The addition of 3 per cent iron to the choline-deficient diet did not result in significant differences in weight. Anemia, as assessed by hematocrit determinations at the time of sacrifice, was not found in any group, and did not account, therefore, for iron deposits in rats fed a choline-deficient diet alone, or for the excessive absorption and hepatic cell deposition of iron in rats given the choline-deficient diet with 3 per cent iron. As determined by thymidine utilization for DNA synthesis in autoradiographs and by hepatic cell mitotic activity, the addition of iron did not cause an increase in regeneration. In animals fed the choline-deficient diet alone, as well as in those fed the diet with added iron, a marked increase in regenerative activity of hepatic cells began when they became markedly fatty, and persisted through the development of the cirrhotic process. This has been described in more detail elsewhere.[63,64] In rats restored to a diet of laboratory meal after various periods on the choline-deficient diet with iron, the amount of regenerative activity, assessed by these methods, was normal.

Experiment II

As shown in Table III, rats previously on a choline-deficient diet for 85 days without added iron, and restored to a diet of laboratory chow for 21 days, when fed laboratory meal with 3 per cent iron did not absorb and deposit significantly more iron in the liver than animals maintained previously on a normal diet. Slight differences were not significant by the t test at the 0.01 level. This would appear to indicate that the choline-deficient diet caused no permanent alterations in the liver, gastrointestinal tract, or other organs that led to continued iron

absorption. Histologically, the livers of rats formerly on the deficient diet showed slight portal and central fibrosis, without cirrhosis. There

TABLE III

IRON CONTENT OF THE LIVER IN RATS FED LABORATORY CHOW WITH 3 PER CENT IRON FOR 40 DAYS, SHOWING LACK OF EFFECT OF A PREVIOUSLY FED CHOLINE-DEFICIENT DIET

	No. of rats	Average wt. of liver at sacrifice (gm.)	Hepatic nonhemin iron *		
			µg. per gm. of liver	Mg. per 100 gm. of liver	Total hepatic iron (mg.)
Group I:					
Previously fed a choline-deficient diet for 85 days, then laboratory chow for 21 days	8	17.5 ± 2.2 †	223.5 ±150.8	22.3 ±15.1	3.8 ±2.4
Group II:					
Previously fed a diet of laboratory chow	8	15.0 ± 1.6	184.5 ± 56.4	18.5 ± 5.6	2.8 ±0.8

* Iron content was determined in 0.5 gm. of liver.
† Standard deviation.

were occasional persistent fatty cysts, slight deposits of ceroid, and focal infiltrations of mononuclear cells. Stainable iron was present in portal areas in fibrous tissue, and in occasional Kupffer cells. Histologically, the gastrointestinal tract was normal.

DISCUSSION

The significance of the present investigation is the demonstration that the same diet which causes cirrhosis may also lead to excess iron absorption from the diet. It also appears that relatively minor alterations in the diet, or in its consumption, determine whether the end picture will be one of pigment cirrhosis with iron deposits in many organs of the body, analogous to hemochromatosis, or iron deposits without pigment cirrhosis, resembling hemosiderosis. The factors causing excess iron absorption with the deficient diet used in this study have not been determined. It was apparent that it was not due to anemia or structural changes in the liver, gastrointestinal tract or other organs. It was probably not the result of choline deficiency because the addition of choline did not protect against the excess absorption. Indeed, the amount of choline added did not prevent a slight degree of fatty infiltration of the liver. The occurrence of cirrhosis was not essential to the excess absorption of iron. In experiments not reported here in detail, iron was histologically demonstrable in hepatic cells after two weeks on the de-

ficient diet, at which time the liver cells contained only small globules of fat. In other experiments, it was found that phosphate deficiency was not a factor, for the addition of even large amounts of phosphate to the deficient diet did not prevent iron deposition in tissues.

Some evidence for the view that iron does not cause tissue damage was obtained in autoradiographic studies. The utilization of tritiated thymidine for DNA synthesis was not greater in hepatic cells containing iron than in normal hepatic cells. Other tissues of the body were also examined in autoradiographs. These included the heart, pancreas, spleen, bone marrow, gastrointestinal tract, kidney, lung, and adrenal; no evidence of increased utilization of thymidine by parenchymal cells was found. The alterations observed in animals in this study were similar to those of human hemochromatosis in that there were pigment cirrhosis and parenchymal iron deposits in the stomach, liver and kidney. There was no excess of iron in the bone marrow. There were a number of differences from the human condition. No small granules of hemosiderin were present in the cytoplasm of bile duct epithelium or pancreatic fibrosis, and iron was not apparent in pancreatic acinar cells before large quantities had accumulated in the adjacent interstitial tissue. No iron deposit appeared in the cytoplasm of myocardial elements. These differences may be attributable to the fact that these were relatively short-term experiments; with studies of longer duration, more comparable to the prolonged disease in human subjects, hemosiderin may be found in these locations. It will also be of interest to learn whether in similar animals, changes can be produced in the serum iron content and serum iron binding capacity by altering the diet and the administration of iron, problems which are relevant to the disease in human beings.

The present experiments are interpreted to support the concept that hemochromatosis and hemosiderosis are variants of a single disorder.

Summary and Conclusions

1. A review of attempts to reproduce hemochromatosis experimentally in animals indicates that these appear to have been unsuccessful, in that the essential lesions of hemochromatosis, pigment cirrhosis and pancreatic fibrosis, have not been produced.

2. In the present investigation, experiments are reported in rats in which a choline-deficient diet which produced cirrhosis of the liver also led to excess absorption of iron and deposition in many tissues of the body.

3. After fatty liver, cirrhosis, and iron deposits were induced by feeding a choline-deficient diet with added iron, animals were fed laboratory chow for several days to weeks before sacrifice. Fat was mobilized from

the liver, leaving a portal type of cirrhosis with hemosiderin deposits, analogous to that seen in human pigment cirrhosis. When a normal diet was fed before cirrhosis had developed, iron deposits were found in a nonfatty and noncirrhotic liver, a condition analogous to hemosiderosis in humans.

4. Autoradiographic studies of DNA synthesis, using tritiated thymidine, indicated that there was no increase in parenchymal cell regeneration in the liver or other organs containing iron.

5. The observations in this study are interpreted to support the concept that in human subjects, hemochromatosis and hemosiderosis are variants of one disease.

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Technical assistance was given by Helen Cadegan, and Jane Donelan. Encouragement and technical help was given by Dr. Thomas Chalmers.

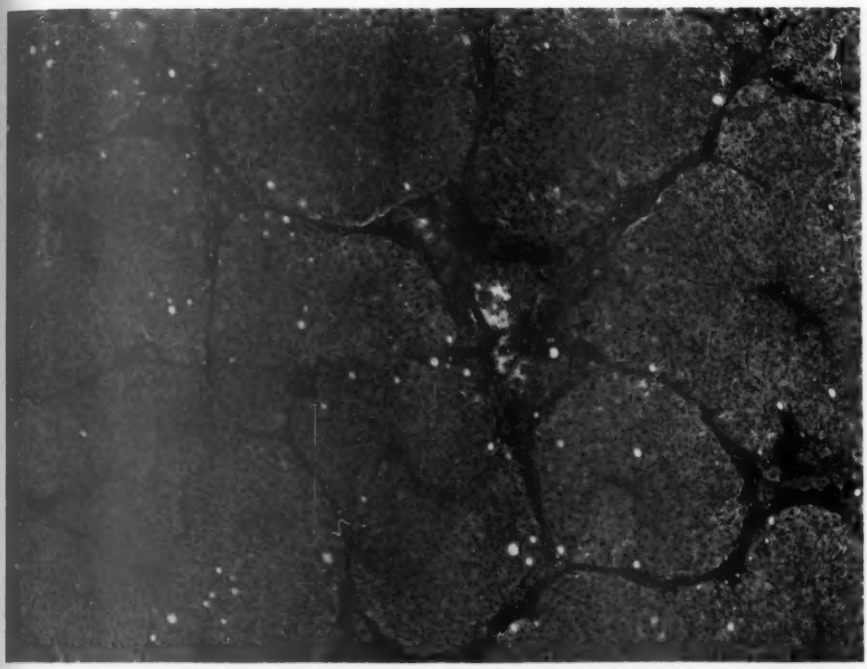
[*Illustrations follow*]

Legends for Figures

Photographs were prepared from sections stained for iron content.

Fig. 1. Hepatic lesion analogous to human pigment cirrhosis. Liver of a rat fed a choline-deficient diet plus 3 per cent iron for 97 days, then restored to a normal diet for 42 days. There is an early, predominantly portal type of cirrhosis with scattered fat globules. Iron is present in large quantities in hepatic cells and in connective tissue. × 80.

Fig. 2. Liver of an animal similar to that shown in Figure 1. A nodular area of relatively nonpigmented hepatic cells is shown, although there are masses of iron in Kupffer cells in the nodule. In adjacent liver cells the uniform intracellular deposits of iron are indicated by the dark color of the cells. Slightly above center is a portal area, with masses of iron around and adjacent to a bile duct. × 800.



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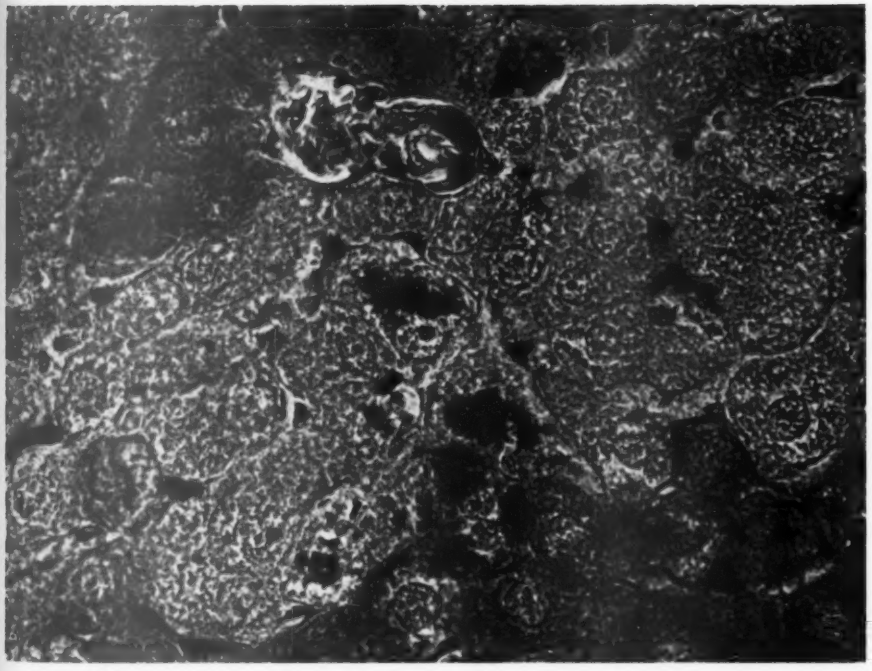

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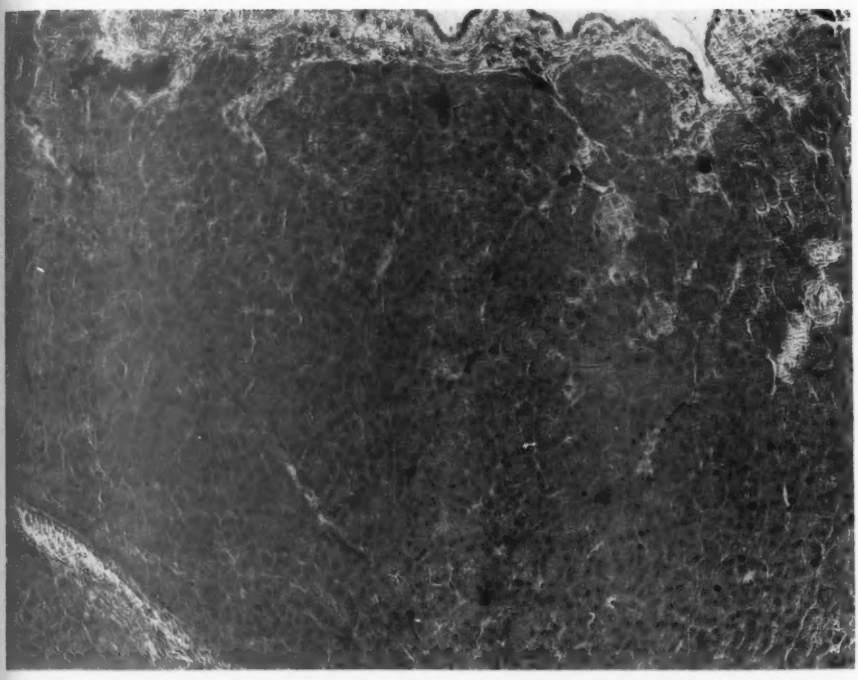
FIG. 3. Pancreas of the rat shown in Figure 2. There are iron deposits, chiefly in interstitial and connective tissue. There is no increase in fibrous tissue. × 200.

FIG. 4. Hepatic lesion analogous to human hemosiderosis. Rat was fed a choline-deficient diet plus 3 per cent iron for 69 days then restored to a normal diet for 26 days. Cirrhosis had not yet developed; except for scattered fat globules, the pattern is one of a noncirrhotic liver with iron in hepatic cells and portal connective tissues. Iron was also present in other organs. × 200.



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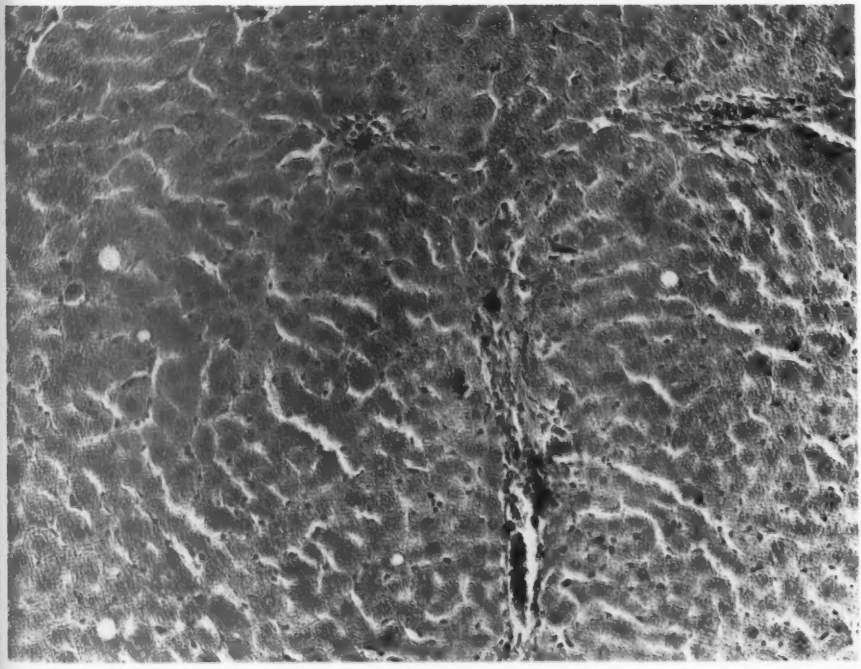

4

BIOCHEMICAL CHANGES IN CARBON TETRACHLORIDE FATTY LIVER

Concentration of Carbon Tetrachloride in Liver and Blood

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Christie and Judah,[1] and Dianzani[2-5] have pioneered in the application of modern biochemical methods to the investigation of the hepatocellular lesions occurring in experimental liver injury. However, the main conclusion reached by these workers—viz., that the primary action of a hepatotoxic agent such as carbon tetrachloride is to cause a breakdown of mitochondrial function in the liver cells—has not been confirmed in a number of laboratories. Calvert and Brody[6] found very definite fatty infiltration of the liver within 5 hours after the administration of carbon tetrachloride; yet these same workers found it difficult to observe functional alterations in the mitochondria before 20 hours. Intraperitoneally administered ethylenediamine tetraacetate (EDTA) provided partial protection against the disturbances in mitochondrial function seen at 20 hours, but fatty degeneration was nevertheless observed. In an interesting study of biochemical changes in experimentally induced liver injury, Neubert and Maibauer[7] found in carbon tetrachloride poisoned rats that a microsomal enzyme catalyzing the detoxication of aminopyrine was strongly depressed whereas mitochondrial oxidative phosphorylation remained intact. In a series of investigations[8-10] carried out in this laboratory on biochemical alterations in carbon tetrachloride poisoning, the development of marked pathologic changes in the liver mitochondria has been amply confirmed. However, the time of onset of the increase in liver fat consistently precedes by many hours the time of onset of the mitochondrial alterations. One of the difficulties in this complex problem is the uncertainty as to the optimum time following intoxication when the experimental measurements should be made, especially with reference to biochemical studies designed to reveal the earliest changes at the enzymic level.

From this point of view, it appeared to us that a study of the concen-

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tration of carbon tetrachloride in the liver as a function of the time following administration would serve as a useful frame of reference for the orientation and interpretation of studies of morphologic and biochemical changes accompanying the development of the hepatic lesions. This communication presents the results of such a study.

Experimental Methods

Determination of Carbon Tetrachloride

A number of analytic procedures have been described for the quantitative estimation of carbon tetrachloride and other halogenated hydrocarbons.[11-14] These methods are based on the colorimetric determination of the Fujiwara chromogen formed when carbon tetrachloride in pyridine is heated in the presence of aqueous sodium hydroxide. Initially, we experienced poor replication and wide variation in color development from day to day. The difficulties were traced to the instability of the Fujiwara chromogen, the marked effects of impurities on the course of the reaction, and the volatility of carbon tetrachloride. The final procedure developed was as follows:

Pyridine (Baker Analyzed Reagent, 5 pounds) was redistilled from 30 gm. of sodium hydroxide (Mallinckrodt Analytical Reagent pellets), until colorless. Sulfur-free toluene was prepared by extracting 10 volumes of toluene (Merck reagent grade) with 1 volume of concentrated sulfuric acid 3 times. The toluene was then redistilled twice, first with anhydrous potassium carbonate and then from calcium hydride. Acetone (Merck reagent grade) was redistilled, first from potassium permanganate and then from potassium carbonate. The pyridine, toluene and acetone were stored in dark bottles. The carbon tetrachloride used was Mallinckrodt analytical reagent (low sulfur).

Carbon Tetrachloride Standard Solution. An aqueous carbon tetrachloride reference solution was necessary to carry out recovery experiments. The reference solution was prepared by adding 10 ml. of carbon tetrachloride below 100 ml. of de-ionized water in a glass-stoppered bottle, previously cleaned with dichromate-H_2SO_4. Use of detergents yielded unreliable reference solutions, presumably due to fine aqueous carbon tetrachloride emulsions. Jarring or agitation of the solution was avoided. Diffusion equilibrium at the above ratio of carbon tetrachloride to water was reached in 72 hours. Reference solutions prepared by vigorous mixing of 10 ml. of carbon tetrachloride and 100 ml. of water, although they eventually reached the same concentration as reference solutions prepared by diffusion equilibrium, were not used since they steadily declined in apparent carbon tetrachloride concentration for 7 days. Reference solutions prepared by diffusion equilibrium were stable

for 4 weeks (the longest period tested). The reference solutions were stored in the dark at 20° C. The concentration of carbon tetrachloride at this temperature was taken as 0.8 mg. per ml. of aqueous phase. The solubility of carbon tetrachloride changes approximately 1 per cent per degree C. in the range 0° to 37°; therefore, small changes in the temperature of the carbon tetrachloride reference solution can be tolerated.

Colorimetric Procedure

The color reaction was carried out in 50 ml. glass-stoppered Pyrex Erlenmeyer flasks. Additions were made in the following order: 10 ml. of pyridine; 1 ml. of water*; 3 ml. of 35 per cent potassium hydroxide w/w prepared immediately before each analysis; 0.5 ml. of toluene; 0.15 ml. of acetone; 0.05 to 0.5 ml. of aqueous carbon tetrachloride standard, corresponding to 0.04 to 0.4 mg. of carbon tetrachloride. For unknowns, the carbon tetrachloride enters the reaction in the 0.5 ml. of toluene addition. The toluene, acetone, and carbon tetrachloride standard were added well beneath the surface of the mixture in the flasks, which were kept stoppered between additions. The Fujiwara chromogen was developed by heating at 70° C. for 40 minutes in a water bath equipped

TABLE I

STABILITY OF FUJIWARA CHROMOGEN

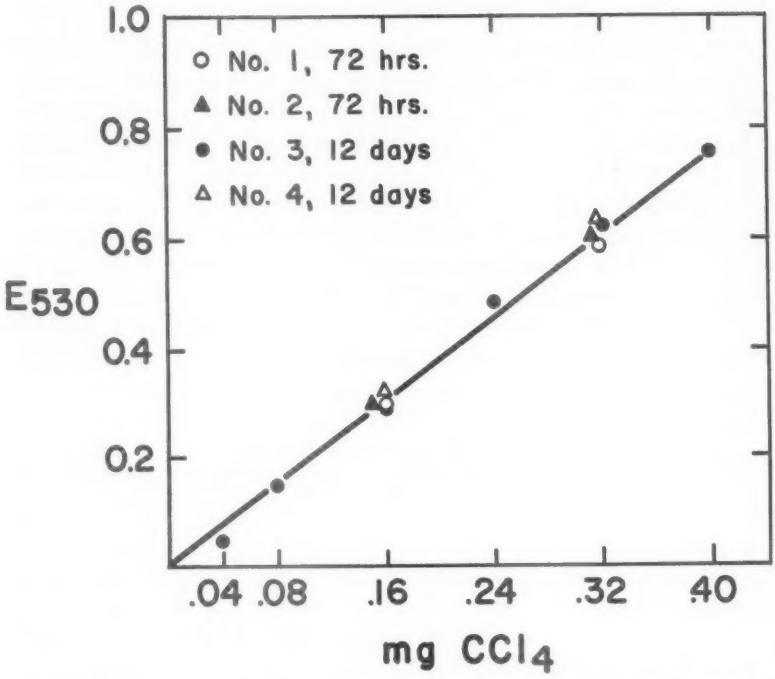
	Optical density at 530 mμ		
CCl_4 mg.	Initial reading	1 hour	3 hours
Blank	0.010	0.000	0.000
0.08	0.180	0.170	0.155
0.08	0.190	0.190	0.180
0.16	0.360	0.360	0.333
0.16	0.395	0.390	0.360
0.32	0.720	0.750	0.700
0.32	0.770	0.700	0.640
0.40	0.875	0.860	0.800
0.40	0.900	0.860	0.800

Conditions: Color developed as in Colorimetric Procedure. Initial readings were taken immediately after mixing 2 ml. aliquots of the pyridine phase, containing the chromogen, with 0.5 ml. of 0.01 N NaOH in the colorimeter tubes.

with a shaker (3.8 cm. excursion, 90 cycles per minute). The red color develops in the upper pyridine phase. After incubation, the tubes were placed in an ice bath, the contents transferred to 22 by 150 mm. glass-

* For analysis of reference solutions, the total volume of water per aqueous carbon tetrachloride reference solution was 1 ml.

stoppered test tubes, and the phases allowed to separate. Two ml. of the upper phase were added to 0.5 ml. of 0.01 N sodium hydroxide in 13 by 100 mm. culture tubes calibrated to serve as colorimeter tubes. The contents of the colorimeter tubes were mixed and the tubes stoppered with corks. Optical density was read at 530 mμ in a Coleman Jr. spectrophotometer, Model 6A.



TEXT-FIGURE 1. Beer-Lambert plots for 4 carbon tetrachloride standard solutions. The elapsed times for preparation to analysis are indicated. Each point represents the mean of duplicate samples.

Stability of the Fujiwara Chromogen. In the absence of added alkali, samples of the final chromogen dissolved in pyridine frequently faded and turned yellow. The fading reaction occurred much more rapidly with stirring or if air was blown through the solution. Stabilizing conditions for the Fujiwara chromogen were achieved by mixing the pyridine phase containing the chromogen with a small amount of alkali, and by keeping the tubes stoppered. The data of Table I indicate the complete absence of color in the blank when read against water, and the reproducibility of the method on duplicate samples from the same carbon tetrachloride reference solution.

Comments on Analytic Procedure

Color development was incomplete when the concentration of potassium hydroxide in the 3 ml. addition of alkali was below 30 per cent or greater than 40 per cent. The observation [12] that addition of a small amount of acetone greatly enhances color development has been confirmed. A concentration of acetone higher than that recommended will depress color development. Since toluene enters the final reaction mixture from the Conway microdiffusion employed for the analysis of carbon tetrachloride in biologic material (see below), the effect of this additive was investigated. It is a fortunate circumstance that 0.5 ml. of toluene, which is a convenient aliquot in the Conway microdiffusion method, proved to be optimum for development of the Fujiwara chromogen under conditions recommended. Heating above 70° or for less than 40 minutes was suboptimal.

Reproducibility and Range of Analysis of the Method. Linearity between color density and carbon tetrachloride content was observed when 0.04 to 0.4 mg. of carbon tetrachloride are present during the chromogenic reaction. Analysis of 4 separate aqueous carbon tetrachloride reference solutions is given in Text-figure 1. It is evident that the preparation and analysis of standard aqueous solutions of carbon tetrachloride by the method described is an adequately reproducible procedure for study of the carbon tetrachloride content of tissues.

TABLE II

RECOVERY OF CARBON TETRACHLORIDE FROM LIVER HOMOGENATES BY CONWAY MICRODIFFUSION METHOD

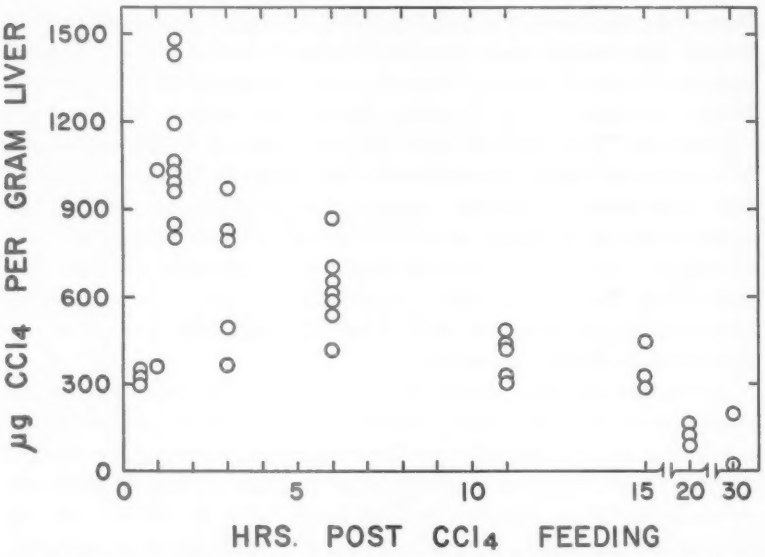
CCl_4 added (μg.)	Number of determinations	CCl_4 recovered (μg.)	Per cent recovery
160	4	66	41
320	15	156	49
640	15	358	56
800	13	438	55
		Weighted mean recovery:	52%

Conditions: Known amounts of carbon tetrachloride were added as aqueous reference solution to liver samples from normal rats, homogenized, and carried through the analysis.

Extraction of Carbon Tetrachloride from Liver and Blood

Food was removed from male rats (The Holtzman Company, Madison, Wisconsin) 10 to 12 hours before carbon tetrachloride feeding. A 1:1 mixture of carbon tetrachloride in mineral oil was administered via stomach tube at a dose of 0.5 ml. of the mixture per hundred gm. of body

weight. Three weighed samples of liver (0.5 to 1 gm.) were taken from each rat. Each sample of liver was homogenized for 30 seconds in 3 ml.



TEXT-FIGURE 2. Concentrations of carbon tetrachloride in rat liver. The values found at each time period were compared with the values found at 1.5 hours in the ranking test (T test) of C. White as given in Snedecor.[20] The corresponding probabilities were: 0.5 hours, $p = 0.01$; 1 hour, $p > 0.05$; 3 hours, $p < 0.05$; all subsequent times, $p \leq 0.05$. Each point represents the mean of 3 determinations on a single rat.

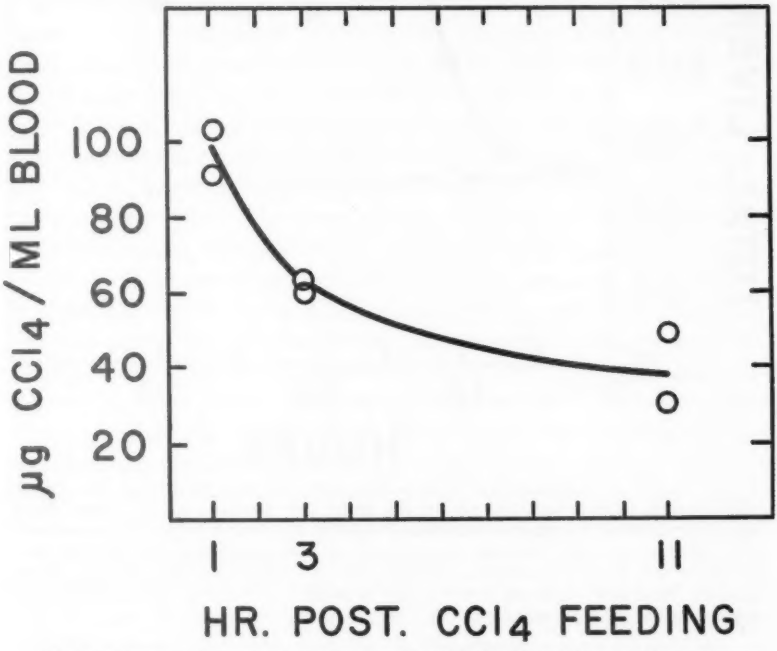
of water in a Virtis 45 homogenizer. The resulting homogenate was transferred to the outer well of a Conway microdiffusion cell[15] which contained 1 ml. of toluene in the center well. The cells were sealed immediately with gum tragacanth paste[16] and gently shaken at the lowest possible speed of an Eberbach shaker (62 cycles per minute, 3.8 cm. excursion). Diffusion equilibrium was reached in 2 hours. A 0.5 ml. aliquot of the toluene phase was analyzed for carbon tetrachloride. The data of Table II show that there was an overall mean loss of 48 per cent when known amounts of carbon tetrachloride, added as aqueous carbon tetrachloride reference solution, were carried through the entire homogenization and microdiffusion procedure. The data of Text-figure 2 have been corrected for this loss, most of which occurs during the homogenization step. For analysis of carbon tetrachloride in blood, 2 ml. of blood were removed from the dorsal aorta in a heparinized syringe and added to 2 ml. of water in the outer well of a microdiffusion cell containing 1 ml. of toluene in the center well. Further procedure was as for liver. The mean recovery of known amounts of carbon tetrachloride

from blood in 18 tests was 81.9 per cent (standard deviation 8.96). The data of Text-figure 3 have been corrected for this loss.

Results

Content of Carbon Tetrachloride in Rat Liver

The time course of the rise and fall of the liver content of carbon tetrachloride following intubation into rats is shown in Text-figure 2. The data indicate that the content of carbon tetrachloride in the liver reached a maximum 1.5 hours following intubation. In control experiments it was shown that the recovery of carbon tetrachloride from homogenates



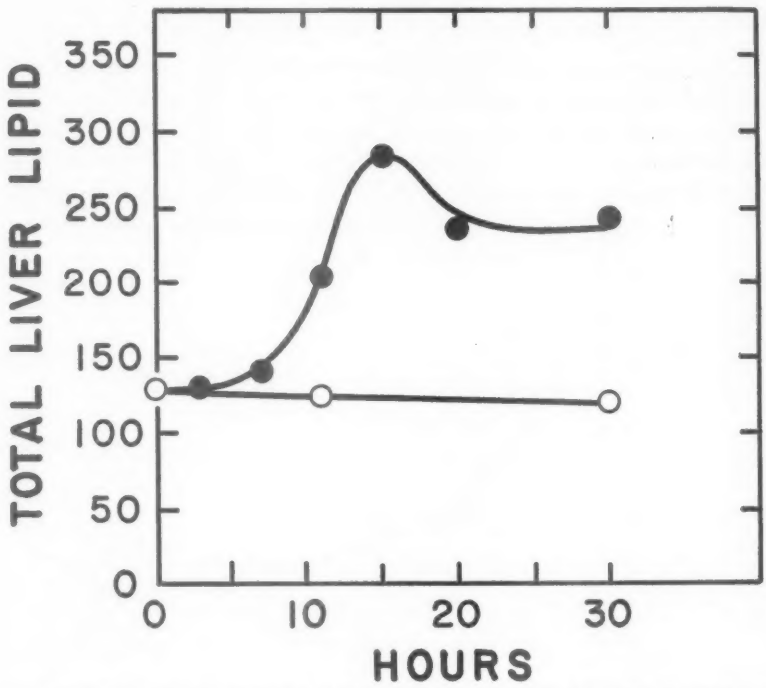
Text-figure 3. Concentration of carbon tetrachloride in rat blood. Each point represents the mean of duplicate determinations on a single rat.

of fatty livers was equal to that from normal livers; therefore, the decline after 1.5 hours is real and not due to failure to recover the carbon tetrachloride because of the increasing fat content. Furthermore, the drop in carbon tetrachloride concentration is too abrupt in comparison to the gradual rise in the liver content of fat.

Content of Carbon Tetrachloride in Rat Blood.

Data on the content of carbon tetrachloride in rat blood are shown in Text-figure 3. Comparison of the data in Text-figures 2 and 3 reveals

that at 1.5 hours carbon tetrachloride is concentrated in the liver approximately 13-fold with respect to arterial blood.



TEXT-FIGURE 4. Rise in liver lipid following carbon tetrachloride feeding. The ordinate represents mg. of total liver lipid per 100 gm. of rat. For the rats fed CCl_4 (upper curve), each point represents the mean of data from 3 rats. For the rats not fed carbon tetrachloride, the number of rats used were: zero, 4 rats; 11 hours, 2 rats; 30 hours, 1 rat. All lipid determinations were done in duplicate or triplicate by the method of Folch, Lees and Sloane-Stanley.[24] Food was withdrawn from the rats receiving no CCl_4 10 to 12 hours before zero time, as in the CCl_4 fed rats.

Rise in Lipid Content of the Liver

In Text-figure 4 are shown data on the rise of the lipid content of the liver in the rats used in these studies. In a subsequent study in which a minimum of 6 rats was analyzed at each time interval, the decline at 20 hours was not observed.

DISCUSSION

The significance of the data presented in this communication is that they form a basic frame of reference, in terms of which studies relevant to the underlying biochemical mechanisms involved in carbon tetra-

chloride fatty liver may be evaluated. For example, biochemical tests for mitochondrial damage based on failure of pyridine nucleotide-dependent oxidations, uncoupling of oxidative phosphorylation (measured indirectly), and a transformation in adenosine triphosphatase properties, have shown [1,9,17] that gross loss of mitochondrial function does not occur until about 20 hours after carbon tetrachloride feeding. A physiologic test system, viz., the ability of potassium depleted mitochondria to reaccumulate potassium,[10] has also indicated that mitochondrial degeneration does not set in until many hours after the peak level of carbon tetrachloride in the liver has been reached. If carbon tetrachloride *per se* is the toxic agent, then the data of Text-figure 2 suggest that the primary hepatic lesion occurs very early following carbon tetrachloride feeding. On the basis of these considerations, the theory [2,3] that loss of mitochondrial function is the key lesion must be seriously doubted.

A variety of studies do indeed indicate that biochemical and physiologic changes occur early following carbon tetrachloride administration. India ink fails to enter the hepatic sinuses and centrilobular veins within the first 2 to 4 hours [18] following subcutaneous injection of carbon tetrachloride into rats. This observation, along with other considerations, led to the theory [18] that vascular involvement and centrilobular ischemia were the key changes in carbon tetrachloride hepatotoxicity. However, evidence against the vascular theory has come from more recent studies of hepatic blood flow measured in unanesthetized, carbon tetrachloride-poisoned rats by the use of a heated thermocouple.[19] These investigations showed that there was no reduction in hepatic blood flow during the period when carbon tetrachloride was producing necrosis. Other evidence for and against the vascular theory has been reviewed.[20] Rosin and Doljanski [21] reported that as early as one hour there was evidence that the centrilobular parenchymal cells were free of pyroninophilic granules, suggesting a loss of cytoplasmic ribonucleic acid. A statistically significant increase in liver fat was observed in rats 3 hours following carbon tetrachloride feeding.[9] An increase in liver weight was evident at 4.5 hours. Further support for the view that the primary biochemical lesion occurs very early following carbon tetrachloride administration comes from the work of Oberling and Rouiller,[22] who examined hepatic cytologic changes following carbon tetrachloride poisoning with the electron microscope. Although some alterations in the mitochondria were noted, these authors emphasized that during the early stages of the lesion, the action of carbon tetrachloride on the mitochondria was much less marked than on the ergastoplasm. The experiments of Neubert and Maibauer,[7] which showed a marked decline in a microsomal enzyme in carbon tetrachloride-poisoned rat liver at a time when mitochondrial

function appeared to be normal, complement at the enzymic level the morphologic observations of Oberling and Rouiller. These observations strongly suggest that the endoplasmic reticulum is one of the earliest subcellular structures to be pathologically altered in carbon tetrachloride poisoning. By comparison of the midpoints in the curves for carbon tetrachloride content as compared to lipid content, the rise in lipid lags about 9 hours behind the rise in carbon tetrachloride. Whatever the primary biochemical lesion proves to be, it appears evident to us that studies of possible derangements in the normal pattern of hepatic physiologic and biochemical functions, suspected of being decisive with respect to the increase in fat, should be carried out during and immediately following the period when the peak concentration of carbon tetrachloride in the liver is reached.

Summary

The concentration of carbon tetrachloride in the liver and blood of the rat following oral administration has been determined. The concentration of carbon tetrachloride in the liver rises rapidly to a maximum level at 1.5 hours, then falls continuously. The maximum concentration in the liver at 1.5 hours is 10 times the concentration at 20 hours. At its maximum concentration in the liver, carbon tetrachloride is concentrated 13-fold with respect to the amount present in arterial blood. The significance of these findings for the relationship of mitochondrial damage to carbon tetrachloride fatty liver is discussed.

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GEOGRAPHIC PATHOLOGY
CHRONIC MYOCARDITIS OF VENEZUELA

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Soon after his arrival in Caracas in 1936, Rudolf Jaffé[1] noted the frequent occurrence of an unusual form of chronic myocarditis of unknown etiology (chronic myocarditis of Venezuela or *miocarditis idiopatica venezolana*) which was a principal cause of death. This condition has also been reported from most of the other Latin-American countries[2] as an important leading cause of death.[3-5] The purpose of the present study was to review the clinical and pathologic features of this disorder in Venezuela and to derive, if possible, suggestions that might lead to an understanding of its etiology, and possibly to its control, prevention or eradication.

Method

Through the courtesy of Dr. José A. O'Daly, Professor of Pathology and Director of the Institute of Pathologic Anatomy of the Central University of Venezuela; Dr. Luis M. Carbonell, Professor of Pathology, Central University of Venezuela, and Dr. José I. Baldó, Chief, Department of Adult Hygiene and Chronic Diseases, Republic of Venezuela, arrangements were made to examine patients hospitalized with the disease. The opportunity was also provided to investigate the available pathologic material pertaining to chronic myocarditis at the Central University and in a number of hospitals in Venezuela. The pathologic material comprised the protocols and prepared slides in various departments of pathology, representative of approximately 100 cases, and fresh tissue from 6 necropsies. Available blocks of myocardial tissue were sectioned and stained with the hematoxylin and eosin, van Gieson, and orcein stains.

Clinical Features

This form of chronic myocarditis in Venezuela affects principally persons of poor economic status from rural areas. Many patients come

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from regions in which Chagas' disease is endemic or give a history of having had the disease. The average age of 42 patients with chronic myocarditis at Vargas Hospital, Caracas, studied by Bruni Celli,[6] was 44 years (range of ages, 18 to 77 years). Most of the patients with the disorder in Valencia, upon whom Brass[7] performed necropsies, were in the fourth or fifth decades of life, a lesser percentage in the sixth or seventh decades.

The disease may cause sudden, unexpected death while the person is at work or walking in the street. More often the patient has one or more episodes of congestive heart failure which, if uncomplicated, generally improve with rest in bed.[8] The failure may last several weeks or months but is likely to recur. The patient's blood pressure is generally normal but may be either mildly elevated or, more often, depressed. On radiologic examination, the outlines of all the chambers of the heart are seen to be greatly enlarged, and the lungs are congested; the enlargement of the left atrium resembles that seen in mitral stenosis. Among the disturbances of conduction revealed by the electrocardiogram are bradycardia, paroxysmal tachycardia, multicentric ventricular extrasystoles, bundle branch block (more commonly of the right bundle), and sometimes atrial fibrillation.

Maekelt[9] found the complement-fixation (Machado-Guerreiro) test to be positive in as many as 60 per cent of patients with chronic myocarditis in Valencia; among 381 persons at necropsy, he found positive reactions in 76 per cent of those with chronic myocarditis, and in only 5 per cent of those who did not have this condition.

Xenodiagnosis with the triatomid insect, *Rhodnius prolixus* (the carrier of Chagas' disease in Venezuela), has been found to be positive in as many as 80 per cent of the patients.[7] If the method of xenodiagnosis is employed, care must be taken to distinguish the crithidial forms of *Trypanosoma cruzi* from those of *Trypanosoma rangeli,* a nonpathogenic organism which in Venezuela may affect man and animals, as well as the *Rhodnius prolixus*.[10,11] In the chronic phase of experimental Chagas' disease in guinea pigs and puppies, Pifano[12] found positive xenodiagnosis in 65 per cent of examinations when the insect was given one blood meal, and in 85 per cent of examinations when the insect was given two meals. From his analysis of the findings in clinical cases, he concluded[13] that approximately 60 per cent of patients with chronic myocarditis gave a positive xenodiagnosis after a single test and about 80 per cent gave a positive result after two tests, if an interval of 2 or 3 weeks was allowed between the two blood meals. At the Vargas Hospital, Bruni Celli[6] noted that 50 per cent of patients with chronic myocarditis had positive xenodiagnosis while 70 per cent of those with positive xeno-

diagnosis had chronic myocarditis. A series of 96 patients were given a total of 123 xenodiagnostic tests; among 42 with chronic myocarditis, 21 (50 per cent) had a positive xenodiagnosis, while of 54 patients who did not have chronic myocarditis, only 9 gave a positive finding (16.7 per cent).

A precipitin reaction[14] has not been used extensively. Blood culture and inoculation of blood into laboratory animals (white mice) for recovery of parasites, while helpful in acute Chagas' disease, are not of value in this form of chronic myocarditis.

Pathologic Observations

Coronary arterial atherosclerosis, which is uncommon in the rural areas of Venezuela,[8] is generally absent or mild among patients with chronic myocarditis from the rural areas near Valencia.[7] Pericardial fluid may be increased (1,500 ml. in one reported instance[15]), and occasionally is serohemorrhagic. Gross evidence of pericarditis may be mild, but is usually absent.

The following represent the principal gross and microscopic features in our cases.

Gross Features

The usual cause of death was congestive heart failure. No evidence was present of hypertension or of coronary, valvular, renal or other diseases. The heart was moderately or greatly enlarged, globose, flabby, and had a dull, lusterless appearance. The weight of the heart generally ranged between 400 and 700 gm. (Köberle[16] reported weights of 1,000 gm. and over.) The musculature was turbid and occasionally exhibited focal scarring. All of the chambers were enlarged, and the region of the pulmonary "conus" was frequently dilated. The dilatation of all the chambers was often extreme and usually greater than the degree of hypertrophy. The wall of the left ventricle in the apical region, and at times the subvalvular region of the posterior wall, was thin and showed early aneurysmal dilatation on occasion[17]; these locations and the right atrium and atrial appendage were also preferred sites for thrombosis and subendocardial fibrosis. The mural thrombi of the right side of the heart frequently gave rise to pulmonary emboli; less often, thrombi in the left cardiac chambers yielded renal, splenic, cerebral or other emboli. Often petechial or larger hemorrhages were present in the epicardium or beneath the endocardium, particularly of the right atrium, and occasionally hemorrhage was present in the subendocardial region at the base of the ventricular septum on the left ventricular aspect. Thrombi or scarring of the myocardium or endocardium appeared in any chamber; the

ventricular septum and the ventricles often were severely involved, particularly the apical portions and the papillary muscles. Fibrosis was patchy and scattered but might also be severe and fairly diffuse in a given area.

Microscopic Features

The epicardium often contained focal collections of lymphocytes. A striking feature was the diffuse and heavy infiltration of the myocardium (Fig. 1) with lymphocytes, and, not infrequently, with histiocytes. The cellular infiltrations might be focal or perivascular, but the arteries and arterioles showed no lesions. Some plasma cells, eosinophils or neutrophils were present in certain instances. Occasionally, granulomatous lesions were prominent and were characterized by areas of necrosis and few giant cells. The most constant and conspicuous features were severe passive hyperemia and interstitial edema (Fig. 2). The inflammatory exudate usually affected the walls of all chambers. Many myocardial fibers showed hydropic or fatty alterations, necrosis (Figs. 3 and 4), atrophy (Fig. 5), or compensatory hypertrophy with large or hyperchromatic or pyknotic nuclei (Fig. 6). Often there seemed to be little or no new connective tissue at the sites of recent destruction of myofibers. Later in the course of the disease, fibrous connective tissue was found chiefly in the subendocardial region and the inner third of the myocardium, focally or over wide areas. Foci of subendocardial fibrosis represented old organized mural thrombi in some instances, and fresh or organizing mural thrombi commonly were present. Capillaries or small veins were occasionally occluded by fibrinous thrombi (Fig. 7) which led to focal fibrosis or microscopic infarcts. A characteristic feature of the myocarditis was the diffuse fine fibrosis (Fig. 8) which differed from the usual appearance of "hypoxemic" fibrosis.[18] Focal areas of calcification (Fig. 9) were found in association with necrosis or fibrosis. Rarely, a leishmanial pseudocyst was found within a myocardial fiber.

Discussion

Concepts Concerning This Form of Chronic Myocarditis

Jaffé[1] reported an incidence of 18 per cent of chronic myocarditis at necropsy (between 1936 and 1944) at the Vargas Hospital in Caracas. In about two thirds of the cases so affected, the cardiac lesion was the cause of death. In 450 necropsies he found microscopic evidence of myocarditis which was often associated with syphilis, schistosomiasis or ancylostomiasis. He believed that these infectious processes produced injury and degeneration of the myocardial fibers and that the absorbed necrotic muscle tissue served as a sensitizing substance[19] which caused

an inflammatory reaction when myocardial tissue was subsequently destroyed by these or other agents or their products, whether present in the heart or other organs of the body. He also thought that the allergic mechanism resulted from parenchymal damage to myocardial fibers, owing to nutritional deficiency or absence of vitamin B_1 in the diet.[20-22] Gil Yépez[23] felt that the myocarditis of Venezuela was related to parasitism and nutritional factors.

Laranja, Dias, Nobrega and Miranda[2] believed that this form of chronic myocarditis represented a chronic stage of Chagas' disease. Triatomid insect vectors of Chagas' disease are distributed over an area extending from the southern part of the United States to Argentina.[2] These workers thought that acute Chagas' disease, which occurs predominantly in infancy and childhood and from which most patients recover after a number of months, gradually evolves as the chronic form of Chagas' heart disease, and that these patients may remain infected for the rest of their lives. While recognizing that this etiology is not proved, Pifano,[11-13] Brass[7] and others[3-5,16,24] also favor the concept that chronic myocarditis is a chronic phase of Chagas' disease. In a number of instances[2,16] one or a few leishmanial pseudocysts have been found upon examination of hundreds of serial sections of myocardium of patients with chronic myocarditis. In one of our cases a number of sections contained pseudocysts, and in a second instance a single section contained pseudocysts.

Tejada and Castro[5] encountered 44 cases of chronic myocarditis among 800 necropsies in Guatemala. The chronic myocarditis, which was by far the commonest form of heart disease in Guatemala, was similar in type to that reported from Venezuela and other Latin-American countries. Because of the absence of parasites from myocardial fibers despite intense diffuse interstitial inflammation, and because most patients with myocarditis came from areas in which Chagas' disease was endemic, Tejada and Castro accepted the hypothesis of Jaffé that the myocarditis was allergic in nature. They explained the myocarditis by recurrent infection with the organism of Chagas' disease. As a result of an initial acute attack of Chagas' disease, some myocardial fibers were thought to have degenerated and become sensitized. Auto-antibodies were released from the sensitized myofibers when the patient was subsequently reinfected with *Trypanosoma cruzi*, thus initiating the allergic reaction. Their idea differed from the concept of Jaffé who did not accept Chagas' disease as the cause of the myocarditis.

Brass,[18] on the basis of material in Valencia, attributed death to chronic myocarditis in 43 per cent of adults who came to necropsy, exclusive of those who died from accidental or unnatural causes. Myo-

carditis was most frequent between the ages of 30 and 60 years. The majority of patients affected were poor farm workers and laborers from regions south and southwest of Valencia, while relatively few of those who lived in Valencia suffered from the disorder. In the gross examination of the heart, he often found dilatation of the pulmonary conus, sometimes aneurysm of the left ventricle, especially at the apex, no evidence of pericarditis, and patchy fibrosis of the muscle, which was more easily recognizable in formalin-fixed tissue. Microscopically, the features were those of a nonspecific chronic myocarditis, chiefly with lymphocytic infiltration. About 10 per cent of the cases had granulomatous lesions, areas of necrosis, and occasionally foreign body giant cells. Brass found no statistical relationship to syphilis, schistosomiasis, or undernutrition. Of 182 patients from the zone east of Valencia where bilharziasis was endemic, at necropsy 32 per cent had bilharziasis and 25 per cent had chronic myocarditis; of 138 patients from the zone west of the city, only 0.5 per cent had bilharziasis and 24 per cent had chronic myocarditis; and of 370 patients from the zone south of Valencia, none had bilharziasis and 50 per cent had chronic myocarditis. Brass believed that the method of xenodiagnosis might furnish presumptive evidence, but that it did not constitute proof that the chronic myocarditis was the result of Chagas' disease. He found no evidence of nutritional deficiency to suggest that the myocarditis was related to dietary insufficiency.

Salfelder [25] stated that he had seen only about 25 examples of idiopathic chronic myocarditis in 1,400 necropsies (1.8 per cent) in Merida, Venezuela (situated in the Andes region, 30 miles south of Lake Maracaibo), and in none of these 25 cases was he able to find leishmanias in the myocardium. He believed that this form of chronic myocarditis had no relation to syphilis, that a relationship to Necator infestation was improbable, and that bilharziasis was not a factor since it did not occur in this locality. Salfelder thought that Chagas' disease might possibly be the basis of the infection, since most of his patients had positive xenodiagnosis. Most of the patients with chronic myocarditis were not cachectic, and he did not regard malnutrition as a factor.

Köberle [16] investigated 100 cases of chronic myocarditis at necropsy. Among the first 50 cases, in each of which as many as 500 serial sections of heart muscle were examined, he found 18 with leishmanial pseudocysts in myocardial fibers. He noted inflammatory cell involvement of the cardiac ganglia and conduction system (sinus node, bundle of His and bundle branches) with destruction of cells or fibers and replacement by fibrous tissue. This was felt to explain the excessive dilatation and hypertrophy of the heart, particularly of the right atrium and right ventricle. Köberle designated this form of cardiac hypertrophy as

"neurogenic." He believed that the chronic myocarditis represented chronic Chagas' disease.

Distinction from Certain Other Forms of Chronic Myocarditis

Dietary Insufficiency. Toreson [26] reported diffuse isolated myocarditis at necropsy in a 15-year-old girl with a long history of dietary insufficiency and malnutrition. The heart weighed 295 gm., the ventricles were hypertrophic, small mural thrombi were present in all chambers, the myocardium was gray-brown, mottled and soft, and showed fibrosis. The myocardial lesions consisted of widespread and irregular hydropic degeneration of muscle fibers, small focal areas of acute inflammation with pronounced exudation of neutrophils, severe edema, hyperemia, and some necrosis of muscle fibers. There were also atrophy and hypertrophy of myocardial fibers as well as replacement by dense collagenous tissue.

Generally, vitamin deficiency is attended by little or no inflammatory infiltration in the myocardium. In beriberi heart disease, the heart is enlarged, but the myocardial alterations are nonspecific, with hydropic degeneration of muscle fibers and interstitial edema.[27,28] Follis [29] stated that in beriberi the heart is reported to be grossly dilated, sometimes hypertrophied, and microscopically to have hydropic degeneration, mild scarring and fatty degeneration. Follis [30] also stated that the anatomic cardiac features in beriberi are equivocal; he noted the complete lack of any consistent pathognomonic lesion at necropsy. An instance of prolonged myocardial disease attributed to beriberi, observed intermittently over a period of 18 years, was reported by Jervey [31] in a 33-year-old Negro woman who drank alcohol excessively and had a deficient diet. Her heart weighed 530 gm., all chambers were dilated, and the ventricles were hypertrophic. The microscopic lesions included interstitial edema, fine fibrosis, some endocardial fibroelastic thickening, but only a rare interstitial collection of leukocytes.

In pigs dying with thiamine deficiency, Follis, Miller, Wintrobe and Stein [32] found cardiac dilatation without hypertrophy, and focal and diffuse myocardial necrosis. In animals that had several episodes of severe thiamine deficiency, scars were present at the sites of healed necrotic lesions. In rats and hogs with deficiency of both vitamin B and potassium, Thomas, Mylon and Winternitz [33] found pronounced cellular infiltration, chiefly of mononuclear cells, in addition to necrosis of muscle fibers.

Sensitization Produced by Drugs. The myocarditis associated with sulfonamide sensitivity [34,35] is attended by focal paravascular or diffuse infiltrations of acidophilic histiocytes with a variable number of other mononuclear cells, both neutrophils and eosinophils. Epinephrine [36] and

l-norepinephrine[37] are other commonly used drugs which may produce myocarditis.

Infectious Diseases. Myocarditis is found most often in association with infectious diseases.[38] In a study of tissue reactions in fatal cases of *Streptococcus hemolyticus* infection, Mallory and Keefer[39] found cardiac lesions in 46 of 79 cases (57 per cent), generally in the form of focal accumulation of cells, mainly lymphocytes and plasma cells, but sometimes neutrophils and eosinophils. They believed that the acute type of lesion with neutrophilic leukocytes may have been induced by localization of bacteria, and that the mononuclear reaction represented a later healing phase of the lesion. The myocarditis, however, was generally focal and typically subendocardial rather than diffuse.

In diphtheria, the "myocarditis" represents part of a reparative process, and fibrosis follows necrosis and destruction of muscle fibers by the diphtheria toxin. Interstitial edema and interstitial infiltration with inflammatory cells may be absent. Gore and Saphir[40] found serous myocarditis in 10 per cent of 160 patients who had had acute or subacute glomerulonephritis; in the affected hearts, the most prominent feature was interstitial accumulation of fluid while the cellular components, chiefly lymphocytes and histiocytes (endothelial leukocytes), were relatively sparse.

Viral Diseases. Myocarditis of nonspecific type has been described in association with a variety of viral affections, such as viral pneumonia, poliomyelitis, and Coxsackie virus infection. Spain, Bradess and Parsonnet[41] reported a patient who died of poliomyelitis after an illness of 2 days with acute myocarditis characterized by interstitial edema, fibrin, focal myocardial necrosis, and focal and diffuse collections of neutrophils and lymphocytes. In 2 patients, each of whom died of poliomyelitis after an illness of 22 days, there was considerable myocardial fibrosis and cellular infiltration consisting principally of lymphocytes. Similarly, Saphir[42] noted, in patients who died of poliomyelitis during the first week of infection, that the myocardium was heavily, and sometimes massively, invaded by neutrophils while in those who died 4 to 6 weeks after the onset of the ailment, the myocardium contained many lymphocytes and monocytes. Ludden and Edwards,[43] in a study of 14 cases of myocarditis associated with poliomyelitis, pointed out the presence of muscular degeneration which included swelling of fibers, loss of striations, cytoplasmic vacuolation, fragmentation, karyolysis, and complete focal necrosis of muscle fibers with irregular staining and disorganized masses of coagulated cytoplasm. These features were present in addition to infiltration of inflammatory cells. Saphir[44] and his associates[45,46] have stated that in various forms of viral myocarditis, in addition to the inter-

stitial exudate, necrosis of isolated fibers or groups of muscle fibers invariably occurs.

Schmidt [47] experimentally produced viral myocarditis in laboratory animals (mice, guinea pigs, and hamsters). In mice, 6 days after infection, the myocardium exhibited small necrotic foci with interstitial monocytic infiltration of surrounding areas, and edema; at 9 days, necrosis was more extensive and fibroblasts began to appear, and these were present in increasing numbers on the eleventh or twelfth day. Calcification was manifest as early as 10 days and was common after the twelfth or 13th day. At 20 days both fibrosis and calcification were present.

Javett and associates [48] investigated an outbreak of acute Coxsackie group B virus infection affecting 10 newborn babies in a maternity home. Six of the babies died of circulatory collapse, and necropsies were performed in 3 cases. In these the heart showed patchy interstitial edema, pleomorphic cellular infiltrations (patchy in 2 and diffuse in 1) with the heaviest involvement in the inner third of the myocardium. The infiltrates consisted of many large mononuclear cells with vesicular nuclei, some with vacuolated cytoplasm, a moderate number of histiocytes with basophilic cytoplasm, a few lymphocytes, and occasional neutrophils, eosinophils, and plasma cells. Fibroblasts were not observed. The muscle fibers contained areas in which the affinity for Heidenhain's hematoxylin stain was lost. Eventually the transverse striations also were lost, and the muscle fibers disintegrated, leaving behind empty sarcolemmal sheaths. The authors believed that the cellular infiltration preceded the changes in the muscle fibers.

Rickettsial Disease. In various rickettsial infections, including scrub typhus (tsutsugamushi disease), epidemic typhus and Rocky mountain spotted fever, Allen and Spitz [49] noted an apparent state of preservation of myocardial fibers despite intense interstitial inflammatory infiltration. In the exudates, plasma cells, acidophilic macrophages or Anitschkow myocytes predominated. In addition, arterial or periarterial lesions were often present, both in the heart and in many other organs of the body.

Parasitic Infections. Recognition of the parasite in the myocardium and usually also elsewhere in the body is generally necessary for diagnosis. In trichinosis [50] the myocardial lesions were usually focal, and the endocardium and epicardium might also be affected. Occasionally, transient larvae were found, but larvae never encysted in the myocardium. The infiltrations were often granulomatous, with histiocytes predominating, and with lesser numbers of neutrophils, eosinophils, lymphocytes and macrophages, and sometimes plasma cells. A few muscle fibers

were necrotic, and healing occurred with fine fibrosis but without sizable scars.

In toxoplasmosis, the central nervous system and the myocardium were chiefly involved. Pseudocysts in the myocardial fibers were often unassociated with inflammation. The infiltrate, which was likely to be present at some distance from parasitized fibers, was composed of focal coagulative necrosis of muscle, neutrophils, eosinophils and mononuclear cells.[51]

Acute Chagas' disease is a clear-cut entity. It affects children primarily but may be encountered at any age, and most often occurs among persons who live in rural areas in huts having palm-leaf covered roofs in which are sheltered the *Rhodnius prolixus*, a nocturnal blood-sucking triatomid insect. Generally a history is obtained that the patient was bitten by the insect, particularly about the face, and that he subsequently had swelling or ulceration of the affected area (chagoma) and regional lymphadenitis (bubo); often *T. cruzi* is recovered from the blood by culture or after animal inoculation, and the xenodiagnosis and complement-fixation tests are positive. Electrocardiographic changes, including disturbances of conduction, are common. Radiologically, all chambers of the heart are generally greatly enlarged. At necropsy the cardiac chambers are greatly dilated, the muscle is pale, mottled and flabby. Microscopically, cystlike accumulations of leishmanias are present in muscle fibers (Fig. 10), and they usually are not surrounded by inflammatory cells. The infiltrates usually occur near the parasitized muscle fibers and consist of lymphocytes, plasma cells, eosinophils, macrophages and occasionally neutrophils. Infiltrations may be focal but are often diffuse and extremely severe.

Enos and Elton[52] reported one instance of fatal acute Chagas' disease in an 18-year-old white North American soldier stationed in the Panama Canal Zone; he had been on guard duty in a forested area. Brass[7] encountered acute infection chiefly in children. In acute human infection with clinical evidences of cardiac insufficiency, many myocardial fibers contained parasites, and the heart was heavily infiltrated with inflammatory cells but was not scarred. In his experiments with white mice, Brass found that leishmanias were most abundant in the heart during the fourth week; the organisms began to disappear during the sixth or seventh week and had virtually disappeared after about 3 months even though inflammatory cells persisted.

Clinically, chronic myocarditis of Venezuela was recognizable by the occurrence of congestive failure without known etiology, by massive dilatation of the heart without accompanying hypertension, and by frequent electrocardiographic conduction disturbances. Pathologically

also, the gross and microscopic features were distinctive although not specific. The epidemiologic and serologic features and the occasional observations of leishmanias in myocardial fibers all suggested an association with Chagas' disease.

Because of the frequent association of bilharziasis, necatoriasis and syphilis, Jaffé [1] suspected that these diseases were factors in the production of the myocarditis. He believed that as a result of any of a variety of conditions, including hypovitaminosis or avitaminosis B_1, myocardial fibers were injured and became sensitized to the products of myocardial degeneration which might be induced by any agent subsequently. Bilharziasis, necatoriasis and syphilis seldom produce myocardial damage by direct invasion of the causative organism, and it seems doubtful that any of these organisms produces a toxin that affects the heart. In many countries where these conditions are common, this form of chronic myocarditis is rare or absent. Moreover, the myocarditis occurs frequently among individuals who do not have any of these diseases or who come from areas in which bilharziasis and necatoriasis are absent or rare. The assumption that the myocarditis is related to hypovitaminosis or avitaminosis B_1 does not have clinical, experimental or pathologic support. Neither can it be regarded as a form of beriberi since in the latter condition the heart shows little or no inflammatory cell infiltration. While it is possible that deficiency of vitamin B_1 or deficiency of a combination of elements in the diet may be related to the chronic myocarditis, the evidence at hand is not convincing.

On the other hand, much weight must be given to the hypothesis that the cardiac inflammatory change may represent an allergic reaction. Although Jaffé [20] denied that Chagas' disease played any important role in the production of this form of myocarditis, his hypothesis nevertheless admitted the possibility that the lesion could result from recurrent infection with the trypanosome. Thus any one of many agents could sensitize the myocardium so that subsequent damage would release autoantibodies and induce an inflammatory reaction in the heart. However, if this were the case, the same type of chronic myocarditis should occur with great frequency in European countries and in the United States. Probably the specific factor or agent operative in the production of this form of myocarditis does not commonly occur in Europe or the United States.

The great majority of investigators in Venezuela and in other Latin-American countries, who have studied this problem, tend to regard the myocarditis as a chronic or recurrent phase of Chagas' disease. Much presumptive evidence exists to support this point of view: (1) The disease is common in localities in which acute Chagas' disease is endemic,

and rare or absent in areas in which it is absent. (2) Although acute Chagas' disease primarily affects children, chronic myocarditis is seen in adults, many of whom give a history of having had acute Chagas' disease. (3) The clinical, radiologic, and electrocardiographic features with respect to the heart are remarkably similar to those seen in acute Chagas' disease. (4) The frequent positive findings with the complement-fixation test for Chagas' disease, and the method of xenodiagnosis are suggestive evidence. (5) Pathologically, the gross alterations (except for scarring) and the microscopic lesions (except for the rarity of leishmanial pseudocysts in myocardial fibers) are similar to those of acute Chagas' disease.

During recent years, the incidence of chronic myocarditis appears to have diminished in the hospitals of Caracas. This decline is attributed[8] principally to expansion and improvement of hospital facilities in the rural areas, so that most patients now enter the local hospitals instead of going to the capital for treatment. The disease is largely absent among inhabitants of metropolitan Caracas. According to figures released by the Ministry of Health in July 1958, approximately 3,100,000 persons, or one half the population of Venezuela, live in rural conditions.[8]

The thought naturally occurs that the lesion might be viral in nature. The microscopic lesions, while nonspecific, resemble those seen in a number of virus diseases of the heart. The prominent interstitial edema, the character of the inflammatory infiltrate, and particularly the destruction of myocardial fibers, all are compatible with reactions produced by viruses. No reports of attempts to recover a virus from the myocardium of these patients have been encountered. It would appear worth while to examine the myocardium for the presence of a virus as an independent agent or as an agent associated with Chagas' disease.

On the assumption that the infection may represent a chronic form of Chagas' disease, Brass[7] believed that the huts in endemic areas should be sprayed twice a year, allowing an interval of about 4 weeks, since at the time of the first spraying the eggs of the insect vectors might be well hidden and thus not affected, while a second spraying after 4 weeks would probably destroy the new larvae. DDT is said not to affect the *Rhodnius prolixus* but destroys ants which are natural enemies of the triatomids.* Salfelder[25] stated that in the further investigations of the disease, it was of primary importance to establish a laboratory well equipped for bacteriology, serology, and parasitology, with expert personnel, in the region where Chagas' disease was apparently most common (Valencia).

* One of the newer effective sprays is Dieldrin, a chlorinated hydrocarbon insecticide (manufactured by Shell Chemical Corporation, Agricultural Chemicals Division, 460 Park Ave., New York 22, N.Y.).

"Chronic myocarditis of Venezuela" appears to be identical with a form of chronic myocarditis that is endemic, frequent, and of serious public health significance in most Latin-American countries. It would appear, therefore, advisable to consider the clinical, pathologic, and epidemiologic aspects of this disease in its international framework. In this fashion there could be formulated measures for prophylaxis, treatment, experimental study, and control.

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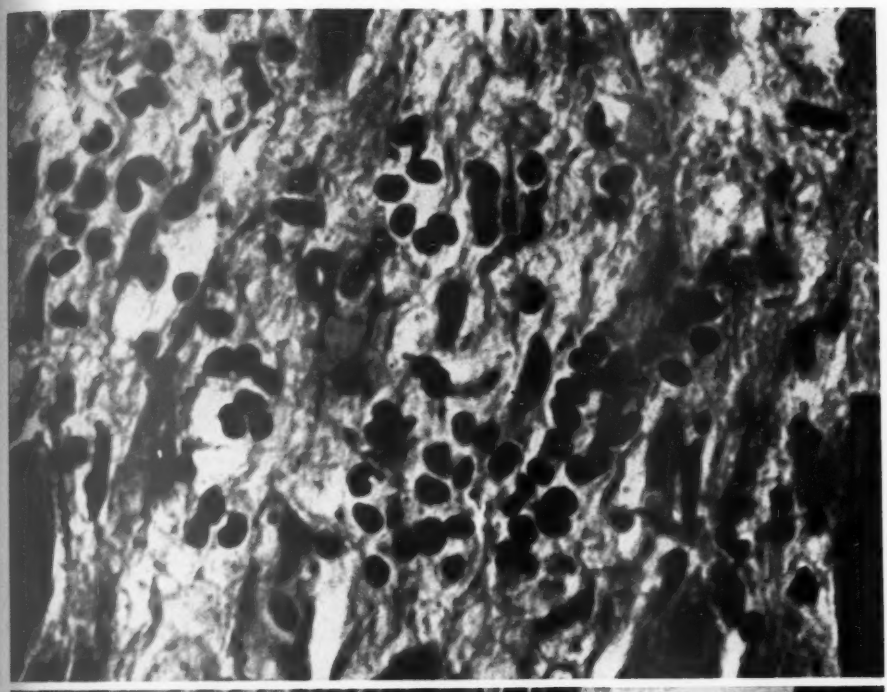
[*Illustrations follow*]

Legends for Figures

Photomicrographs were prepared from sections stained with hematoxylin and eosin.

Fig. 1. (Case 10581, Vargas Hospital, Dr. Bruni Celli.) A 32-year-old woman with congestive heart failure provided a history of having been bitten by a triatomid insect. The complement-fixation test for Chagas' disease was negative. At necropsy there were hydropericardium and bilateral hydrothorax; the heart weighed 650 gm. The myocardium exhibits degeneration or disappearance of myofibers. A serous exudate contains lymphocytes and histiocytes. × 400 (enlarged 1½ times).

Fig. 2. (59B63, 59P103. Case 2110, Children's Hospital, Dr. Lozano.) Chronic myocarditis in a child. Note interstitial edema, hydropic degeneration and disintegration of muscle fibers. × 825.



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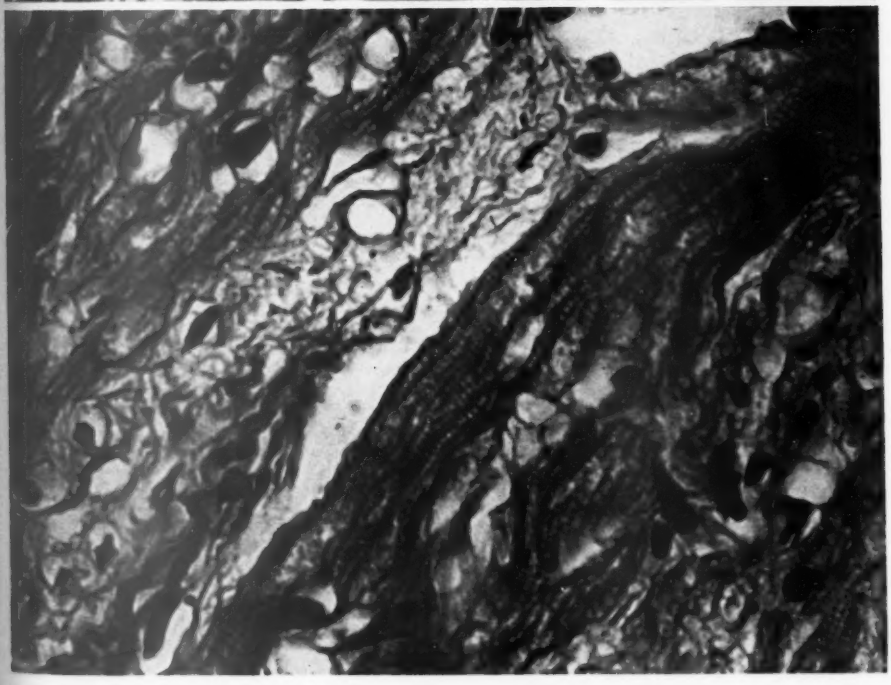

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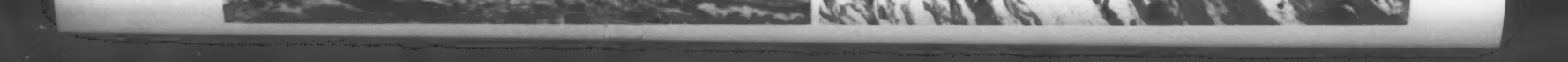
FIG. 3. (Case illustrated in Fig. 1.) Severe degeneration of muscle fibers is accompanied by edema, hyperemia, infiltration with lymphocytes and histiocytes, and some collagenization. There is atrophy of some muscle fibers and hypertrophy of others. × 100 (enlarged 1½ times).

FIG. 4. (Case 10255, Vargas Hospital.) Left ventricle. There is interstitial edema and interruption and loss of some myocardial fibers. Hypertrophy appears in others. Note also the characteristic patchy fine fibrosis. A few collagen fibers are evident. × 100 (enlarged 1½ times).



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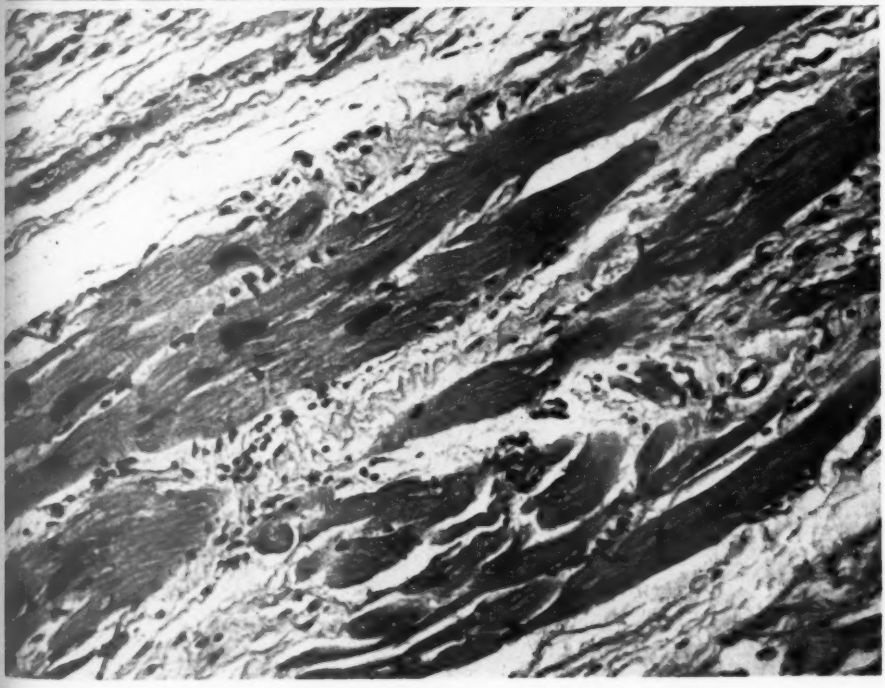

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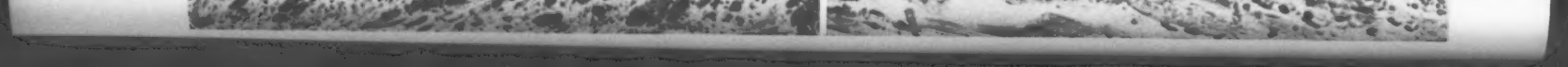
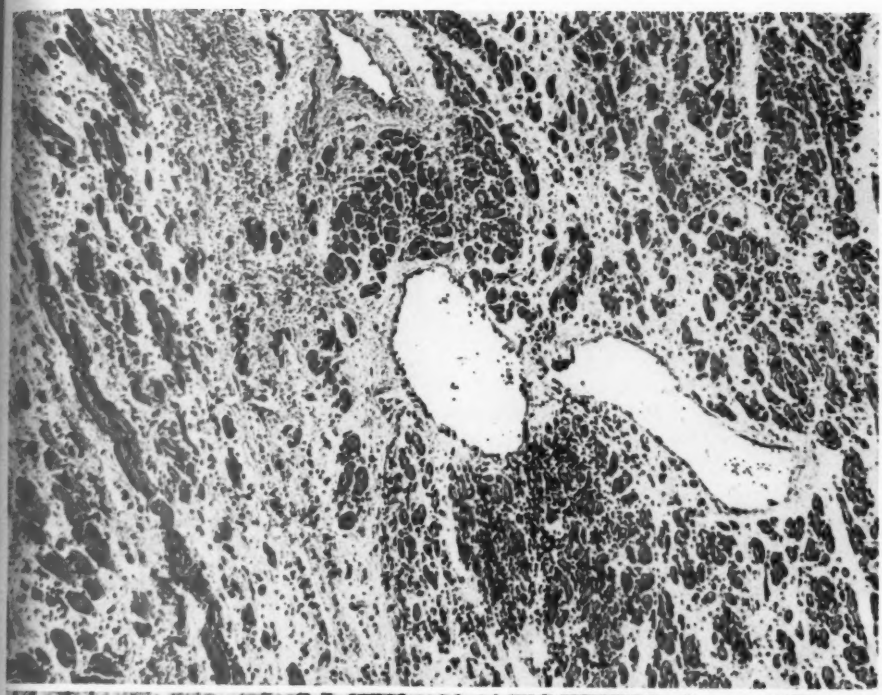
FIG. 5. (Case illustrated in Fig. 1.) Right ventricle. Note focal and scattered lymphocytic infiltrate, dilated capillaries, diffuse edema, atrophy of muscle fibers and, in the left portion of the field, patchy fibrosis. × 30 (enlarged 1½ times).

FIG. 6. (59B67. Case 10512, Vargas Hospital.) A 40-year-old man with a history of Chagas' disease died of congestive failure. Hydrothorax and ascites were present. The heart weighed 700 gm., and all chambers were enlarged ("global dilatation"). The left ventricle contained mural thrombi, and a recent thrombus was present in the right atrium. Focal active chronic inflammation is associated with hyperemia, interstitial edema, hydropic degeneration of muscle fibers, and occasional hypertrophic muscle fibers. × 100 (enlarged 1½ times).





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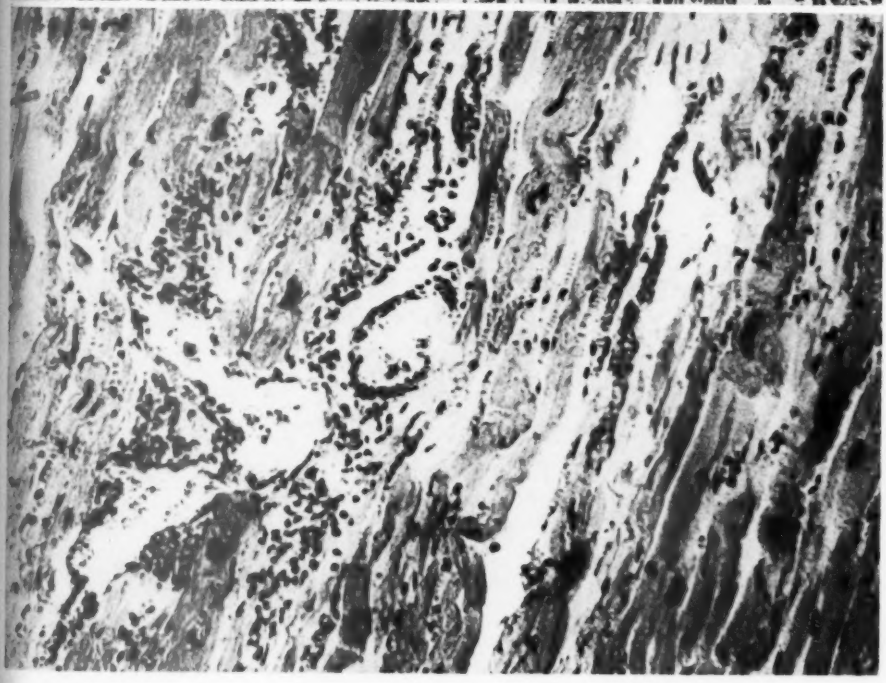

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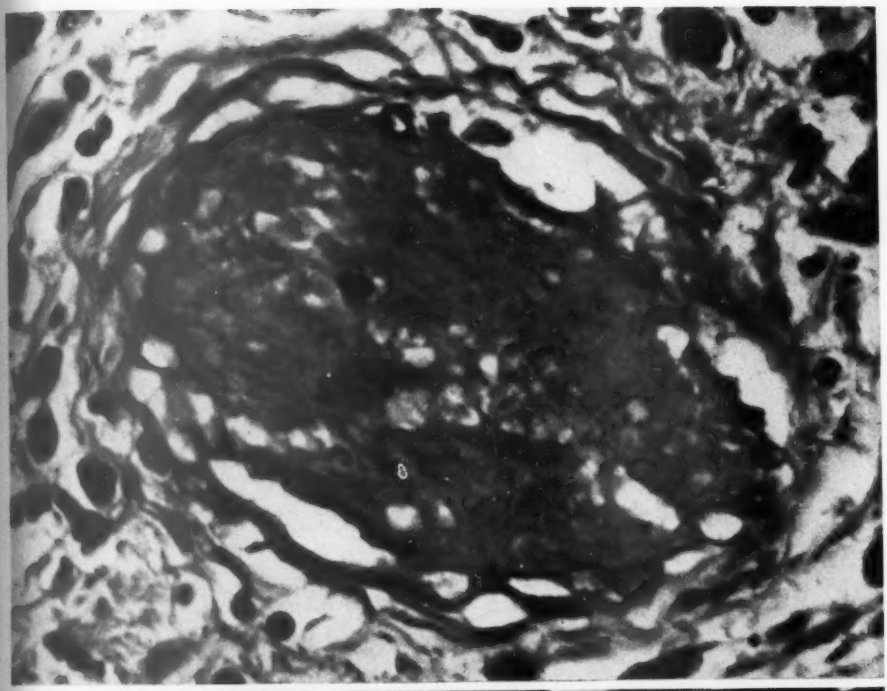
FIG. 7. (Case 9704, Vargas Hospital.) A recent capillary thrombus is shown. There is irregularity, swelling and apparent discontinuity of the capillary wall, and serous exudate around the capillary. × 400 (enlarged 1½ times).

FIG. 8. (A30-59, Central Hospital, Valencia, Dr. Brass.) A 28-year-old man from an area in which Chagas' disease was endemic died suddenly. The heart weighed 440 gm. and had a small aneurysm at the apex of the left ventricle. Extensive patchy fine fibrous connective tissue is frequent in this type of myocarditis. × 160.



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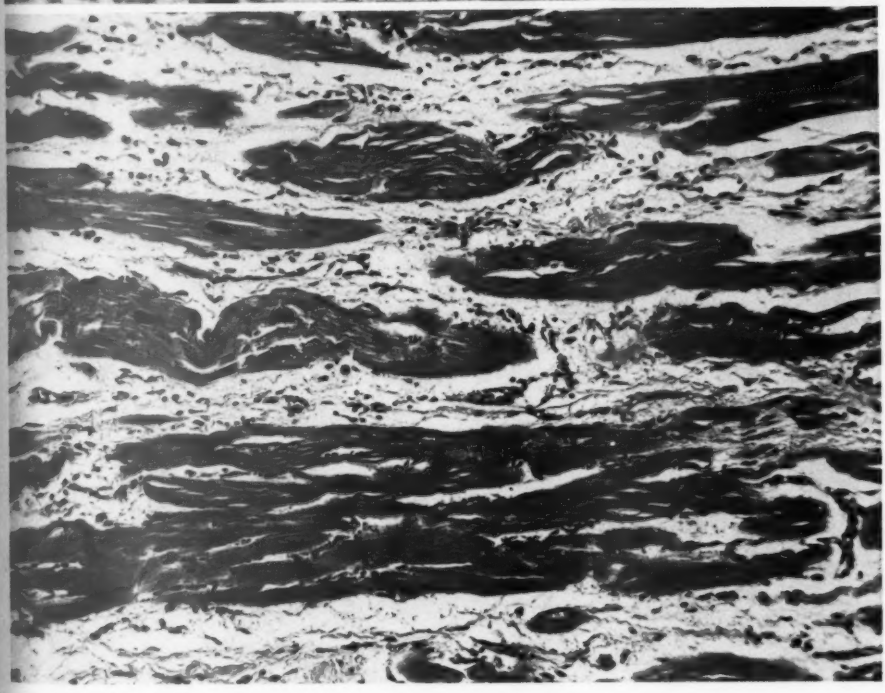

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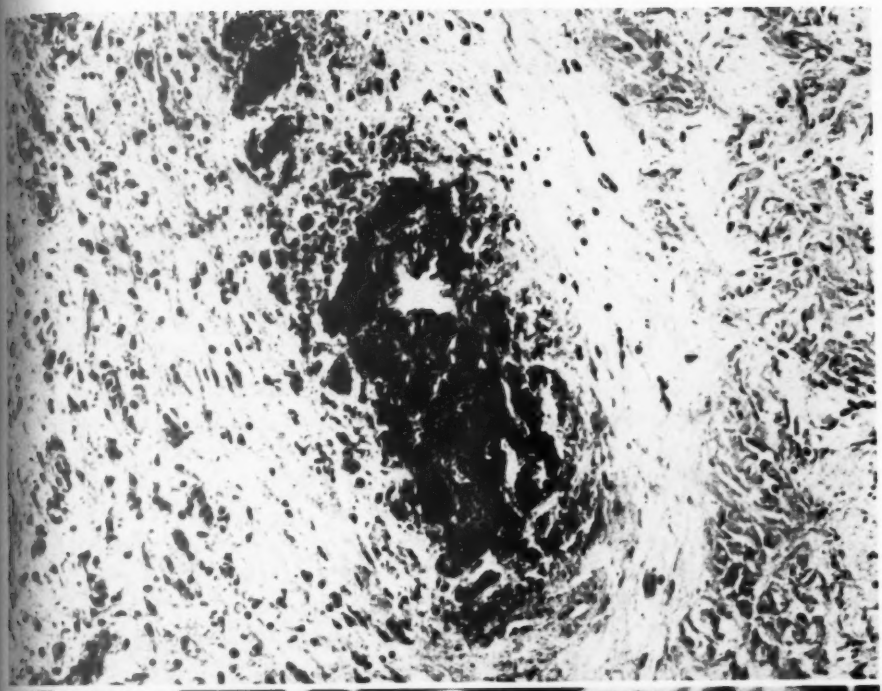
FIG. 9. (Same case as illustrated in Fig. 1.) An area of calcification and edema appears in the center. There is hyaline necrosis of muscle fibers at the right and infiltration with lymphocytes and histiocytes with beginning fibroblastic proliferation at the left. × 100 (enlarged 1½ times).

FIG. 10. (A152-59, Central Hospital, Valencia.) A 4-year-old girl from an endemic area of Chagas' disease, had a history of having been bitten by many triatomid insects. She died of acute chagasic myocarditis and encephalitis a few hours after admittance to the hospital. The heart weighed 130 gm. (normal weight, 72 gm.); all chambers were dilated, and the myocardium was pale. A leishmanial pseudocyst is evident in a myocardial fiber. Adjacent to it are edema and an infiltration, chiefly of lymphocytes and histiocytes. × 860.



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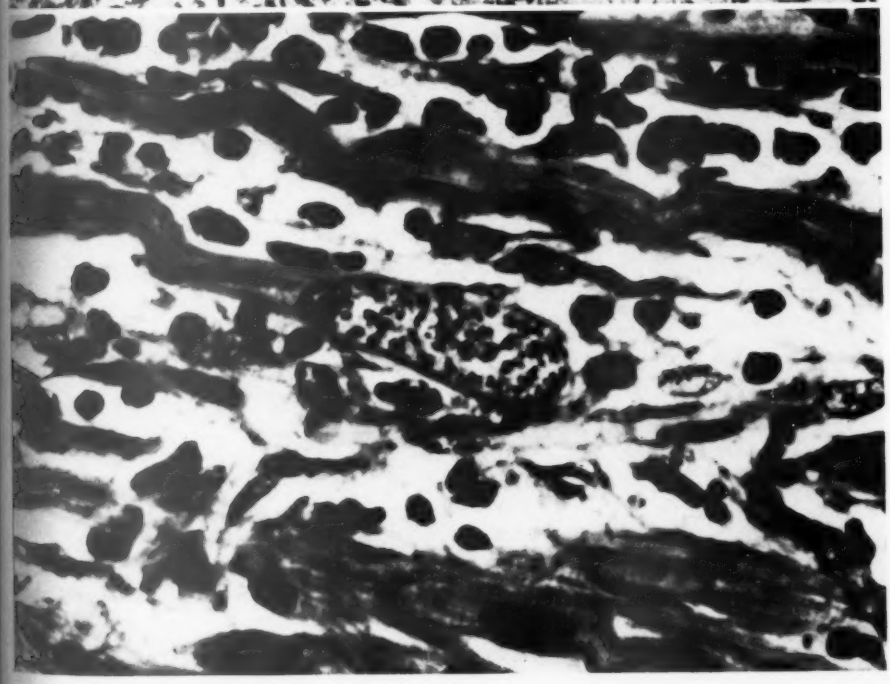

10

GEOGRAPHIC DIFFERENCES IN THE SEVERITY OF AORTIC AND CORONARY ATHEROSCLEROSIS

The United States, Jamaica, W. I., South India, and Japan

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Many of the widely accepted concepts of human atherosclerosis stem from epidemiologic studies and from consideration of startling geographic differences in mortality from coronary artery disease. Aschoff [1] observed a reduced severity of atherosclerosis during the famine that Germany suffered after the first World War. Nutritional factors were considered responsible for the infrequency of atherosclerosis among Chinese,[2] Okinawans [3] and poorly nourished Costa Ricans.[4] Malmros [5] and Strøm and Jensen [6] noted that a fall in mortality from vascular heart disease in the Scandinavian countries during World War II coincided with a decided restriction of dietary fat. Extensive epidemiologic surveys convinced Keys [7] that the fat content of the diet determines the incidence of coronary artery disease through its influence upon the level of serum cholesterol. In contrast to the high prevalence of vascular heart disease in the United States, residents of England, Denmark, and Sweden, and the African Bantu, Japanese natives of Kyushu, and natives of Madrid and Naples enjoy a low incidence of this lethal affliction, presumably because they subsist on a diet low in fat. Racial factors do not seem to be significant. Japanese in Hawaii and Italians in the United States exhibit an increased incidence of coronary artery disease when their dietary regimens alter to approximate those of their adopted countries. Clinical investigation, moreover, has demonstrated that the level of blood cholesterol can be altered by regulating the intake of fat; in this

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connection, however, the quality and nature of the ingested fat seems to be at least as important as its quantity.[8]

These observations are substantial, but they nonetheless constitute indirect evidence, based as they are upon mortality tables and blood cholesterol determinations. Even when allowance is made for the unavoidable errors inherent in mortality tables, such data can only disclose the fraction of a widely prevalent process that for one or more reasons

TABLE I

GEOGRAPHIC ATHEROSCLEROSIS. DISTRIBUTION OF CASES

	Number of cases		
	Aorta	Coronary artery	Myocardial infarcts * (Age 40 or more)
United States	1130	1176	276/1067
Jamaica, W.I.	535	384	7/268
India (Vellore)	258	105	5/105
Japan (Sapporo)	260	258	10/147

* The numerator represents the number of myocardial infarcts; the total group is expressed as the denominator. These figures, limited to individuals over 40 years of age, exclude 3 myocardial infarcts in younger individuals in the United States group.

attains clinical magnitude. In respect to occlusive arterial disease particularly, vital statistics do not seem to distinguish between basic intimal disease and distinctly separate complicating processes to which the lesions may be susceptible. Accordingly, it would be advantageous to secure pathologic data which would permit comparison of atherosclerosis in different geographic areas. Unfortunately, the descriptions of atherosclerosis in virtually all necropsy records are not adequate for this purpose. The pathologic analyses of vascular disease in the South African Bantu[9] and the Japanese in Kyushu[10] suffer for lack of objective methods for assaying and recording the degree of atherosclerosis observed at necropsy. Except for occlusive lesions, there has been considerable and unavoidable inaccuracy in comparing the data of different observers who have not had a common background of experience.

A simple appraisal procedure has been described recently. This has demonstrated its applicability to comparative studies of atherosclerosis in New Orleans, Guatemala, Costa Rica, Los Angeles, and Japan (Sapporo).[11-16] From these investigations it has become increasingly apparent that surveys limited to appraisals of aortic disease may be inadequate to show the basis for a more than threefold difference in the incidence of coronary artery disease in the case of Los Angeles and Sapporo, Japan. Accordingly, arrangements were made to collect quantitative ap-

praisals of aortic and coronary atherosclerosis from 4 widely separated geographic areas: the United States (Boston and Los Angeles); Jamaica, W.I.; Vellore, South India; and Sapporo, northern Japan. With one exception (G.G.H.), all participating members were introduced to the appraisal procedure by the senior author.

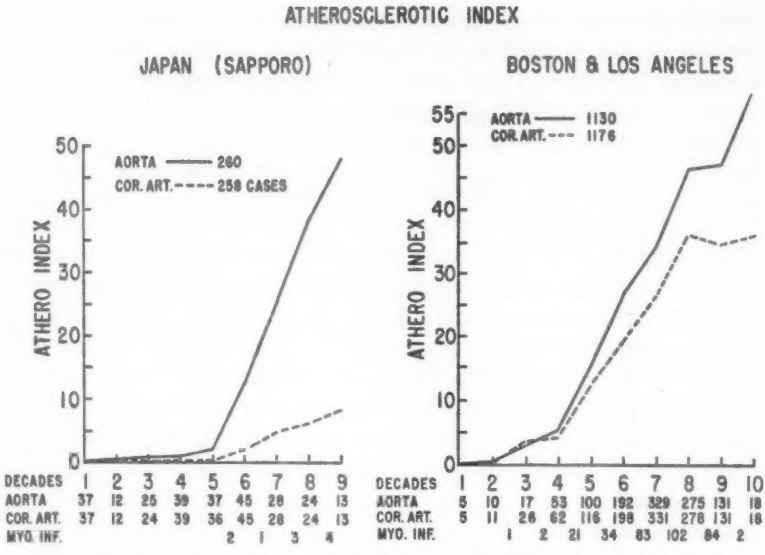
Material and Methods

In each center, tissues were procured from routine unselected necropsies upon both male and female subjects for evaluation of aortic and coronary atherosclerosis. For this purpose, the 3 grossly dissectable coronary arteries were considered as a single channel. Stains were not used to accentuate lipid or other features of the intimal lesions. The number of specimens examined in each of the geographic areas is shown in Table I. The age distribution is indicated by decade in the accompanying Text-figures. As a measure of the relative clinical significance of the vascular disorder in each area, we have listed also the proportion of individuals 40 years of age or older, who had sustained myocardial infarction. Lack of correspondence of the figures for aortic and coronary appraisal is attributable to incomplete examinations and, in the United States material particularly, to the practice of preserving minimally affected aortas for grafting procedures.

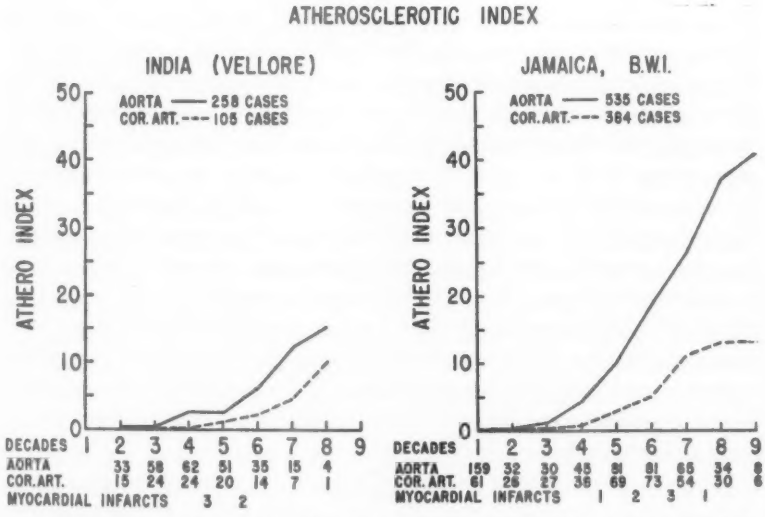
In brief, the quantitative appraisal of atherosclerosis entails estimations of the extent of surface involvement and of the decimal fraction of this involvement comprised by each of 4 types of intimal lesion. Intimal lesions are characterized as: grade 1, lipid streaks, spots, or patches; grade 2, fibrous and atheromatous plaques; grade 3, necrotic, hemorrhagic or thrombotic plaques; and grade 4, calcified plaques. In terms of surface area there were 5 groups; group O included those cases presenting less than 5 per cent surface involvement; group A, 6 to 15 per cent; group B, 16 to 33 per cent; group C, 34 to 50 per cent; and group D, surface involvement exceeding 51 per cent. These features, comprising a 5 digit figure, an "atherosclerotic profile" by appropriate weighting, are converted into an atherosclerotic index expressing severity on a scale ranging from 0 to 100. The weights assigned to each type of intimal lesion vary logarithmically; a value of one is assigned to grade 2, a tenth to grade 1, and 10 to grades 3 and 4. The sum of these weighted values is multiplied by the area factor, a figure which varies directly with the proportion of the intimal surface which bears any type of atherosclerotic alteration.

Results

Changes in the atherosclerotic index with decade increments in age are depicted in Text-figures 1 and 2. During the first two decades and

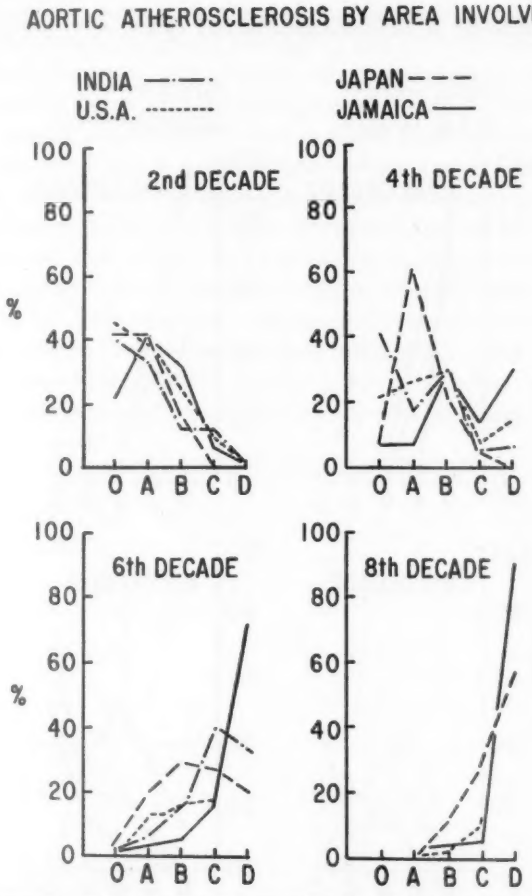


TEXT-FIGURE 1. Progression of aortic and coronary atherosclerosis with age, Japan and the United States.



TEXT-FIGURE 2. Progression of aortic and coronary atherosclerosis with age, India and Jamaica.

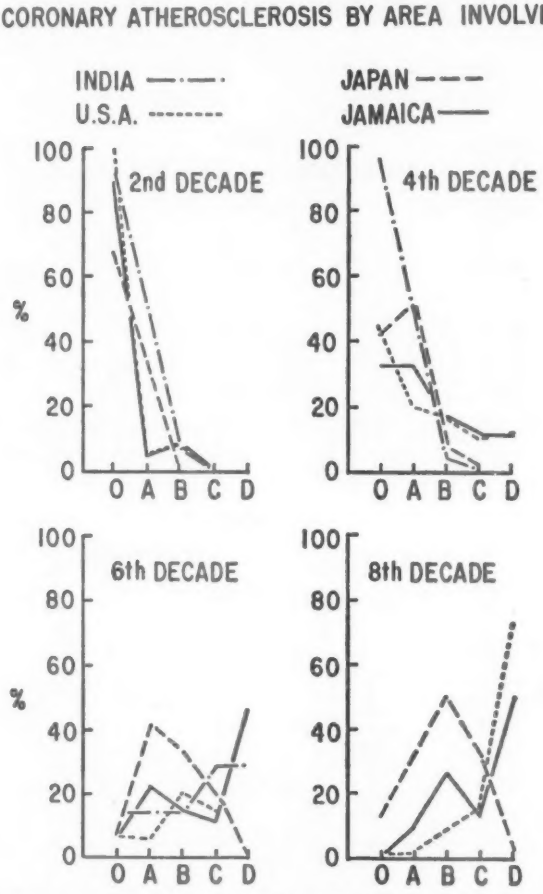
even longer in the Japanese specimens, this figure is insignificant, reflecting the low numerical weight assigned to grade 1 lesions and not an absence of intimal disease. In the subsequent decades the index rises



TEXT-FIGURE 3. Effect of age on surface extent of aortic atherosclerosis, India, United States, Japan and Jamaica.

progressively. Of the 4 geographic areas, the United States displays the most and India the least accretion of aortic atherosclerosis. In Japan and Jamaica the severity of the aortic process approaches that observed in the United States. Coronary atherosclerosis, as seen in the United States greatly exceeds that found in the other 3 areas and develops to an appreciable magnitude approximately one decade earlier, concurrent

with aortic intimal disease. This disparity is least marked in the United States sample and most marked in Jamaica and Japan. Coronary atherosclerosis is of approximately the same order of severity in India, Japan and Jamaica. Commensurately, the occurrence rate of myocardial in-

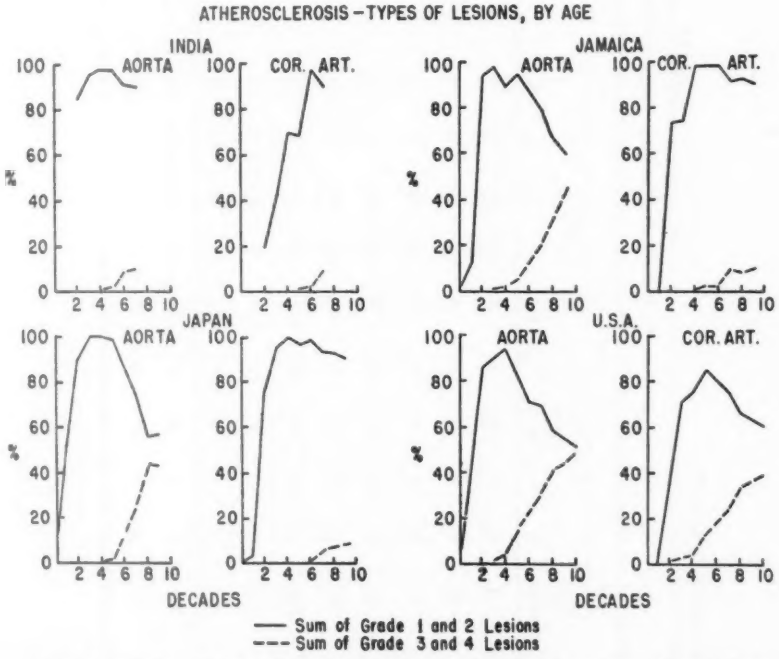


TEXT-FIGURE 4. Effect of age on surface extent of coronary atherosclerosis, India, United States, Japan and Jamaica.

farction (Table I, and Text-figures 1 and 2) in the United States is more than 3 times that found in the other localities. Significance cannot be assigned to the differing rates of incidence of ischemic myocardial damage in Jamaica, India and Japan because of the small size of the samples. It is noteworthy that cases of myocardial infarction were not limited to groups with high coronary atherosclerotic indexes. While most of the

afflicted individuals had more severe atherosclerosis than the average for their age group, some did not, and a few, indeed, had less than average intimal disease.

Graphic representation of the average surface extent of intimal alteration in the second, fourth, sixth and eight decades discloses the increase which is expected with aging (Text-figs. 3 and 4). This extension, however, in both the aortic and coronary areas is noticeably slower in the Japanese group. Consideration of the proportionate involvement by each of the 4 types of atherosclerotic lesions shows major geographic differences in the occurrence of grade 3 and 4 lesions. These values are listed in Tables II to V with accompanying data on area involvement in the corresponding material. For graphic demonstration and for greater accuracy, the values of grade 1 and 2 lesions have been added and plotted according to age and the sums of the figures representing grades 3 and 4 (Text-fig. 5). This expedient was adopted because of the great individual variation in designating lesions as either grade 1 or 2. The identification of complicated lesions (grades 3 and 4) is much more precise, but, not infrequently, ulcerated lesions may also be calcified. With the



TEXT-FIGURE 5. Relative frequency of the types of aortic and coronary intimal lesions with age, India, Japan, Jamaica and the United States.

TABLE II

EXTENT AND SEVERITY OF SURFACE ATHEROMA: THE UNITED STATES

Decade	Aorta (1130 cases)										Coronary artery (1176 cases)									
	No. of cases	Area involved Group					Severity of lesion Grade				No. of cases	Area involved Group					Severity of lesion Grade			
		O	A	B	C	D	1	2	3	4		O	A	B	C	D	1	2	3	4
1	5	5	0	0	0	0	26	16	0	0	5	5	0	0	0	0	0	0	0	0
2	10	5	4	0	1	0	72	14	0	0	11	11	0	0	0	0	27	1	0	0
3	17	4	5	3	1	4	44	46	0	0	26	13	6	3	0	4	31	41	0	2
4	53	11	14	16	4	8	48	46	2	2	62	27	12	10	6	7	39	37	1	3
5	100	9	17	24	18	32	21	62	6	9	116	22	15	16	27	36	26	59	2	10
6	192	3	13	30	32	114	12	59	9	13	198	15	12	40	28	103	19	63	3	15
7	329	2	10	31	44	242	6	63	12	19	331	9	22	40	35	225	10	65	2	23
8	275	0	3	7	32	233	3	56	13	28	278	3	6	24	43	202	5	61	3	31
9	131	0	0	4	10	117	2	53	11	34	131	3	3	12	26	87	4	59	3	34
10	18	0	0	0	0	18	3	48	12	37	18	2	0	2	3	11	4	56	2	38

TABLE III

EXTENT AND SEVERITY OF SURFACE ATHEROMA: JAMAICA, W.I.

Decade	Aorta (535 cases)										Coronary artery (384 cases)									
	No. of cases	Area involved Group					Severity of lesion Grade				No. of cases	Area involved Group					Severity of lesion Grade			
		O	A	B	C	D	1	2	3	4		O	A	B	C	D	1	2	3	4
1	158	154	3	1	0	0	13	0	0	0	61	61	0	0	0	0	0	0	0	0
2	32	7	13	10	2	0	92	2	0	0	26	23	1	2	0	0	73	0	0	0
3	30	2	10	6	8	4	90	7	0	0	27	12	10	2	2	1	70	4	0	0
4	45	3	3	15	6	18	63	16	1	0	38	12	12	6	4	4	90	8	0	0
5	81	1	6	13	13	48	47	48	3	2	69	10	13	16	13	17	79	19	1	1
6	81	1	3	5	13	59	27	60	10	3	72	4	16	11	8	33	68	30	1	1
7	65	0	1	6	5	53	20	59	15	6	54	2	8	8	10	26	59	32	2	7
8	34	0	1	0	2	31	13	54	19	14	30	0	3	8	4	15	53	39	3	5
9	9*	0	0	0	0	9	10	46	27	17	6	0	0	1	2	3	50	40	5	5

* One older than 90 years.

TABLE IV

EXTENT AND SEVERITY OF SURFACE ATHEROMA: INDIA (VELLORE)

	Aorta (258 cases)										Coronary artery (105 cases)									
	No. of	Area involved Group					Severity of lesion Grade				No. of	Area involved Group					Severity of lesion Grade			
Decade	cases	O	A	B	C	D	1	2	3	4	cases	O	A	B	C	D	1	2	3	4
2	33	14	11	4	4	0	76	8	0	0	15	14	0	1	0	0	16	4	0	0
3	58	24	14	15	5	0	90	5	0	0	24	18	2	3	1	0	27	15	0	0
4	62	26	11	18	3	4	83	14	0	0	24	23	1	0	0	0	45	25	0	0
5	51	13	15	15	5	3	71	26	1	1	20	14	2	4	0	0	35	34	0	1
6	35	2	5	10	9	9	55	41	3	1	14	5	5	2	1	1	43	56	1	0
7	15	0	2	2	6	5	44	47	6	3	7	1	1	1	2	2	23	74	0	3
8	4	0	0	0	1	3	20	70	2	8	1	0	0	0	1	0	5	85	0	10

TABLE V

EXTENT OF SEVERITY OF SURFACE ATHEROMA: JAPAN (SAPPORO)

Decade	Aorta (260 cases)										Coronary artery (258 cases)									
	No. of cases	Area involved Group					Severity of lesion Grade				No. of cases	Area involved Group					Severity of lesion Grade			
		O	A	B	C	D	1	2	3	4		O	A	B	C	D	1	2	3	4
1	37	37	0	0	0	0	51	0	0	0	37	37	0	0	0	0	3	0	0	0
2	12	5	5	2	0	0	83	17	0	0	12	8	4	0	0	0	65	10	0	0
3	25	6	10	9	0	0	80	20	0	0	24	13	10	1	0	0	87	9	0	0
4	39	4	24	9	2	0	74	26	0	0	39	16	20	3	0	0	86	14	0	0
5	37	3	14	17	2	1	64	35	1	0	36	9	22	5	0	0	80	17	0	0
6	45	2	9	13	12	9	40	46	8	6	45	3	18	15	9	0	59	40	0	1
7	28	0	2	2	11	13	25	48	14	13	28	2	1	17	6	2	51	43	1	5
8	24	0	0	3	7	14	15	41	18	26	24	3	0	12	8	1	43	50	1	6
9	13	0	0	0	3	10	18	39	21	22	13	1	2	1	8	1	40	51	1	8

lesions plotted in this fashion, it is evident that grade 3 and 4 lesions make their appearance in the fourth decade in the United States and Jamaica, but about a decade later in Japan and India. Thereafter, a progressively greater proportion of the affected population reveal grade 3 and 4 lesions. This progression of the disorder in the aorta and the coronary artery is of small magnitude in the South Indian cases; complicated lesions comprise only 10 per cent of the intimal process in the seventh decade. In Jamaica and Japan, grade 3 and 4 lesions similarly constitute only a minor fraction (9 and 6 per cent respectively) of the lesions in the coronary arteries. By contrast, in the seventh decade, the United States study showed that grade 3 and 4 lesions constituted 31 and 25 per cent of the alterations in the aorta and coronary arteries respectively. Corresponding figures for the aortas in Jamaica and Japan are 21 and 27 per cent.

Discussion

A previous pathologic investigation of a large United States sample has shown that coronary atherosclerosis is generally less severe and progresses with age at an appreciably lower rate than the accompanying process in the aorta.[16] The degree of parallelism was such, however, that in the absence of more direct pathologic evidence, it seemed reasonable to consider appraisals of atherosclerosis in different population groups. Indeed, comparing a United States sample with one from Guatemala had demonstrated a considerable difference in the severity of atherosclerosis parallel to the differing incidence of ischemic heart conditions.[12] However, further comparative geographic studies in Jamaica and Japan failed to show this relationship. In these countries a low incidence of coronary artery disease was accompanied by a disproportionately severe degree of aortic atherosclerosis.[16]

As the present investigation has demonstrated, a much greater disparity between aortic and coronary atherosclerosis exists in Jamaica and Japan than in the United States population where both are severe, and in the Indian group where both are relatively mild. The basis for this difference is not apparent, but since the Japanese residents of Hawaii[7] and members of the Indian armed forces[17] do not exhibit this relative freedom from coronary artery disease, it seems unlikely that racial factors are important. This, indeed, is one of the points of evidence demonstrating the importance of diet, specifically its fat content, to atherosclerosis. Table VI details the average daily consumption of composition of national diets. The values from Japan[18] and the United States[19] are based upon national average food availability data; those from India[20] and Jamaica[21] were taken from two small surveys of in-

TABLE VI

GEOGRAPHIC VARIATION IN DIET

	Calories	Protein	Fat	Carbohydrate
United States	3220	97 gm. (12%)	148 gm. (41%)	384 gm. (47%)
Japan	2025	70 gm. (13.8%)	22.8 gm. (10.1%)	385 gm. (76%)
India *	1724	47 gm. (10.9%)	19 gm. (9.9%)	349 gm. (79.2%)
Jamaica †	2276	65 gm. (12%)	44 gm. (17%)	405 gm. (71%)

* Peanuts are the main source of fat.
† Coconut oil is the principal fat.

dividual food intake. The major differences demonstrated by these data indicate a greater caloric value and a much higher fat content in the United States diet than in the diets of the other 3 nationalities.

While these dietary differences may be correlated with variations in the severity of coronary atherosclerosis, they do not explain why commensurate differences are not evident in the aorta as well. On this point, the impression gained in reviewing this material merits further investigation. Hypertension and cerebral apoplexy seem distinctly more frequent in the samples from Japan and Jamaica than in that from the United States. Is it possible that the augmenting effect of elevated blood pressure upon atherogenesis is greater in the aorta than in the coronary arteries? The data presented in Table VII are equivocal and inadequate to answer this question.

TABLE VII

INFLUENCE OF HYPERTENSION UPON ATHEROSCLEROSIS: JAMAICA

	Hypertensive				Nonhypertensive			
	Aorta		Coronary		Aorta		Coronary	
Decade	No. cases	Atheroma index	No. cases	Atheroma index	No. cases	Atheroma index	No. cases	Atheroma index
40–49	15	15.8	12	6.3	66	8.6	57	1.8
50–59	31	24.3	27	7.7	50	14.9	45	3.5
60–69	16	23.3	15	9.7	49	27.0	49	9.0
70–79	15	39.0	13	13.5	19	34.8	17	12.4
80–89	3	48.4	3	13.8	5	35.8	3	12.6

When the incidence of myocardial infarction is used as an index of occlusive coronary artery disease, it is evident that the frequency of this complication, on the average, parellels the severity of the coronary atherosclerosis. This generalization should not obscure the less frequent occurrence of ischemic myocardial damage with mild lesions of the

intima. Since myocardial infarction did not occur in the absence of some coronary intimal alteration, even seemingly minor degrees of atherosclerosis appear to predispose to thrombosis. This is not necessarily occlusive and, as Duguid[22] has asserted, the subclinical deposition of fibrin upon the intima may indeed be an important factor in atherogenesis. Inquiry into the basis for the occurrence of complicating thrombosis of this nature has led to numerous studies of the coagulation mechanism in clinical atherosclerosis. These, as well as similar inquiries into the influence of dietary fat upon blood coagulation have been inconclusive.[23-25] In view of the restricted localization of occluding thrombi, it is perhaps more pertinent to consider local rather than systemic influences. Early as well as late lesions of atherosclerosis have been associated with localized loss or attenuation of the normal anticoagulant property of the vessel wall.[26]

Summary

Comparison of aortic and coronary atherosclerosis in the United States, Jamaica, Japan and India demonstrates that after the first two decades of life, there is a greater severity of intimal alteration in the United States than in the other population groups. The 3 foreign populations exhibited much less coronary atherosclerosis and a lower incidence of myocardial infarction. Their diets, too, were of lower caloric content and contained less than half the fat found in the United States diet. In Japan and Jamaica, atherosclerosis proved to be disproportionately more severe in the aorta than in the coronary arteries. Accordingly, appraisals of atherosclerosis limited to the aorta do not provide adequate indexes of geographic variations. A much lower proportion of complicated atheromatous lesions (ulcerated or calcified plaques) accounted for most of the differences observed.

While there was a general parallelism between the severity of coronary atherosclerosis and the frequency of myocardial infarction, there were instances of cardiac infarction associated with relatively minor degrees of coronary intimal alteration. These situations indicate that virtually all grossly recognizable intimal lesions may be complicated by thrombosis.

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MYOCARDIAL ISCHEMIA AND EARLY INFARCTION

A Histochemical Study

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This investigation is part of a study of early morphologic changes seen in myocardial ischemia and infarction produced by ligation of the coronary artery of the rabbit. Although the process of myocardial infarction has been extensively studied in both human subjects and experimental animals, knowledge of early morphologic alterations is scanty. It was hoped that with histochemical and electron microscopic techniques, it would be possible to demonstrate the very early sequelae of ischemia and to determine the anatomic alterations which indicate irreversible damage to the myocardium. Progressive changes from 5 minutes to 5 hours after ligation of the coronary artery of the rabbit, as detected by the electron microscope, have been previously reported.[1]

Although most investigations of experimental myocardial infarction have been performed in the dog, some studies have been done with small laboratory animals.[2,3] In the dog there has been wide variation in the immediate postoperative mortality and in the distribution and homogeneity of the infarcts following coronary ligation. For adequate chemical or histologic evaluation, the experimental infarct should be uniform; such infarcts have been produced in the posterior papillary muscle of the dog[4] and in the rabbit.[5] The rabbit was chosen as the experimental animal in the present instance for several reasons. One person, unassisted, could perform the necessary surgical procedures. Animals of uniform age, weight, and physical condition could be obtained readily and at low cost. Transverse sections of the entire heart could be prepared to provide easier distinction of the junctional zones between normal and ischemic muscle.

Material and Methods

The coronary arteries in 14 rabbits were examined grossly and by injection techniques[6,7] to determine the pattern and distribution of the vessels. Hearts injected with vinyl acetate (obtained from Ward's Natu-

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ral Science Establishment, Inc., Box 24, Beechwood Station, Rochester, New York) were digested with concentrated potassium hydroxide to leave a cast of the coronary arteries. Those injected with barium gelatin mixtures were prepared by a flat, unrolling technique [8] which permitted radiologic demonstration of the coronary arteries. A total of 86 albino rabbits, both male and female, weighing 4 to 5 pounds, were utilized in this experiment. Details of the surgical technique employed for ligation of the lateral descending branch of the left coronary artery have been previously described.[1] Anesthetized animals were sacrificed at intervals ranging from one minute to 120 hours after coronary ligation.

All animals were protected from anoxia during sacrifice by positive pressure respiration. Early in the experiment, specimens were obtained by rapid excision of the beating heart; later, in an effort to prevent continued contraction and to reduce the metabolic requirements of the heart, specimens were obtained by rapid removal of the arrested heart. Cardiac standstill was produced within 15 seconds after the intravenous injection of 5 to 10 mEq. of potassium chloride or 10 to 20 mEq. of calcium chloride. Transverse sections of the entire heart were placed in various fixatives which included absolute alcohol and acetone at 4° C., picric alcohol-formalin, cold 10 per cent phosphate-buffered formalin at pH 7, 10 per cent calcium formol, Romeis' and Carnoy's solutions. Many specimens were quenched in isopentane at −160° or in propane at −175° and dehydrated by freeze-drying or freeze-substitution.[9] Tissues were embedded in paraffin and sectioned at 6 μ. Smaller fragments of 43 of these hearts were examined with the electron microscope.

Sections were cut from each heart and were treated with various techniques. The staining methods [10] included hematoxylin and eosin, Gomori's one-step trichrome, and Masson's trichrome stains.

A variety of histochemical methods were employed. Prussian blue reaction for iron,[10] and the murexide [11] and alizarin red S [12] techniques for calcium were used on tissues fixed in neutral buffered formalin. Von Kossa's method for phosphate [13] was used after alcohol fixation. Stains for potassium, by means of a modified cobaltinitrite method,[14] were done on tissues fixed in alcohol and prepared by freeze-substitution. Aqueous and methanol periodic acid-Schiff (PAS) stains [15] were performed following a variety of fixatives. Oil red O and Sudan black B in propylene glycol [16] were used for the staining of fat. In a few animals, stains for succinic dehydrogenase, TPN diaphorase, DPN diaphorase, sulfhydryl and disulfide groups were performed. Since changes were not demonstrated at 12 hours, these techniques were not systematically investigated.

Results

Injection studies revealed certain features common to all hearts examined (Figs. 1 and 2). The first branches of the left coronary artery supplied the basal portions of the septum. After further bifurcation, several branches descended toward the apex. Although the coronary arteries of the rabbit are embedded in the myocardium, they were readily identified, and a branch could be tied midway between the base and apex of the heart. A successful occlusion could be recognized by pallor of the ischemic muscle within 30 seconds, and by cessation of contraction within 1 to 2 minutes.

Three of the 86 animals died of anesthetic accidents before thoracotomy could be performed, and 3 served as mock thoracotomy controls. Ten animals died of ventricular fibrillation during thoracotomy or in less than 15 minutes after coronary occlusion. Sixteen animals were excluded from the study because the ligature included a major vein. In 49 the coronary artery was ligated without inclusion of the vein, and the animal survived for longer than 15 minutes. Five animals died of failure or fibrillation between 15 minutes and one hour. Five animals died spontaneously, at intervals from 7 to 32 hours, from asphyxia caused by obstruction of the tracheotomy tube by clotted blood and mucus. Most of the unsuccessful or unsatisfactory attempts occurred early in the experiment.

Nineteen animals showed varying degrees of myocarditis. Usually this consisted of one or more small, isolated foci exhibiting loss of myocardial fibers, with infiltration by large mononuclear cells or cardiac histiocytes (Figs. 3 and 4). Electron micrographs revealed sharply delineated focal areas of necrosis within some apparently surviving muscle fibers. Animals with more active or more disseminated evidences of carditis tolerated the operative procedure poorly. Seven of the operative and immediate postoperative deaths occurred in rabbits with myocarditis.

In the ischemic region, the earliest and most striking change detected was the rapid loss of glycogen. In tissue fixed in absolute alcohol and stained with PAS, a sharp zone of demarcation between normal and ischemic muscle could be detected 5 minutes following coronary artery ligation (Fig. 5). In a cross section of the entire heart, this usually involved the lateral wall of the left ventricle. Near the apex the ischemic area often extended into the adjacent septum. A layer of cells beneath the endocardium and about major thebesian veins usually retained glycogen (Figs. 6 and 7).

Hearts which were arrested by injection of potassium chloride or calcium chloride showed more glycogen in normal areas than did those

which were excised while beating. In arrested hearts, definite, clearly demarcated regions with partial loss of perinuclear glycogen could be demonstrated within one minute of ligation (Fig. 8). Loss was complete within 5 minutes.

In a study of early lesions following ligation of the circumflex coronary artery in the dog, fresh frozen cryostat sections, post-fixed in alcohol containing one per cent mercuric chloride, made it possible to achieve large, easily oriented sections and accurate cytologic localization without evidence of glycogen flight (Fig. 9). Much glycogen remained in normal muscle after formalin fixation, but greater quantities were present following fixation in cold absolute alcohol. Optimum cytologic preservation and localization of glycogen was obtained by freeze-drying or freeze-substitution of tissue obtained from animals in which cardiac arrest was induced by injection of potassium or calcium chloride (Fig. 10). After the initial loss of glycogen from an ischemic area, a PAS-positive material which was not digested by diastase appeared in the myocardial fibrils (Fig. 11). This material could be demonstrated in tissues fixed in a variety of ways. It was constantly and diffusely present by 12 hours after coronary occlusion and was consistently absent before 60 to 90 minutes. This non-glycogen, PAS-positive material was first detected in very slight quantity at the periphery of 60 minute lesions. Thereafter, it was found in tissue fixed in formalin, Carnoy's, or picric alcohol solutions, more diffusely in the cytoplasm of injured cells throughout the damaged area. It was not noted until 4 to 5 hours after coronary ligation in tissues fixed in absolute alcohol or absolute acetone, or in those prepared by freeze-drying or by freeze-substitution in alcohol.

Another striking difference was noted between the tissues exposed only to dehydration by organic solvents and those subjected to strong protein-precipitant chemical fixatives. After any fixation, PAS stain resulted in coloration of the coarse, cytoplasmic, predominantly perinuclear glycogen, the basement membranes of capillaries, the sarcolemma, the intercalary discs and the Z bands. After chemical fixation, diastase digestion removed only the coarse, perinuclear, presumably labile or unbound glycogen (Fig. 12). However, after absolute alcohol or acetone fixation, after freeze-drying or freeze-substitution in alcohol or acetone, the Z bands no longer stained with PAS following diastase digestion (Fig. 13). The same phenomenon was noted in the polymorphonuclear leukocytes present in the area of infarction; PAS-positive material in their cytoplasm was removed by diastase digestion of alcohol and acetone-fixed material but not by digestion of tissues fixed with chemical protein precipitants.

Deposition of iron, calcium and phosphate, as revealed by Perls's,

murexide, alizarin red S, and von Kossa's stains was first seen as small, widely separated basophilic granules within the cytoplasm of myocardial fibers at the margin of the ischemic zone or adjacent to small blood-filled capillaries within the ischemic zone. No deposits were noted earlier than 4 hours; deposits were minimal and inconstant at 4 and 5 hours after coronary occlusion, but were consistently present at the border of the ischemic areas in 6 hours. At 12 hours or more, the intracytoplasmic granular mineral deposits had become more diffusely distributed throughout the area of infarction but remained most intense at the periphery of the infarct and about vessels.

In confirmation of the experience of others,[17] small quantities of calcium and iron, which could be demonstrated following fixation in neutral buffered formalin, were absent after fixation in unbuffered 10 per cent formalin, because of the solubility of calcium and iron salts in acid media.

Stains for potassium in tissues fixed in absolute alcohol demonstrated the same pattern of flight to the periphery of the cell as did glycogen. Definite evidence of loss of cell potassium was present 21 hours after ligation when the unstained cells were in marked contrast to the heavy staining of potassium in edema fluid.

No evidence of fatty degeneration of myocardial fibers was found in 6 hours or less after coronary occlusion. No animals were examined in the interval between 6 and 12 hours. At 12 hours, results were variable. No fatty change was noted in one animal; two rabbits had foci, most prominent at the periphery of the ischemic area, of diffuse fatty droplet alteration in the cytoplasm. At later stages the number of animals investigated was insufficient to draw conclusions. The most intense fatty degeneration was noted in two animals which died 12 to 20 hours after ligation, following obstruction of the tracheal cannula. Beyond 20 hours, fat droplets became relatively less prominent in myocardial fibers and more prominent in the cytoplasm of phagocytic cells. This remained true 96 to 120 hours after coronary occlusion. In this instance fat droplets were present in the occasional isolated cells surviving in the area of infarction.

As early as two hours after coronary ligation the infarct could be detected in hematoxylin and eosin stained sections by cytoplasmic eosinophilia of damaged fibers and by perivascular infiltration with polymorphonuclear leukocytes.

Discussion

The heart is remarkably versatile in its ability to adjust coronary flow and oxygen consumption in response to hypoxia[18] and to utilize a wide variety of substrates for its metabolism.[19] It is richly provided with

mitochondria for oxidative metabolism and with cytoplasmic enzyme systems for anaerobic glycogenolysis. If glucose or glycogen becomes unavailable, it can utilize in increased amounts lactic acid, pyruvic acid, fatty acids or ketone bodies. If oxygen becomes unavailable, contraction can continue only so long as active phosphate groups can be provided by anaerobic glycogenolysis. The cessation of contraction in ischemic myocardium observed within one to two minutes after coronary occlusion probably represents the completion of glycogenolysis and the unavailability of active phosphate groups for muscle contraction or for oxidative phosphorylation.[20]

The anticipated very early loss of glycogen has been demonstrated in this study by electron microscopy [1] and by stains for glycogen with light microscopy. Rapid loss of glycogen [21-23] and accumulation of lactic acid [21] have been demonstrated chemically [24,25] in anoxic hearts. Histochemical studies of infarction have indicated glycogen loss in 30 to 60 minutes.[26,27]

The method of animal sacrifice has a significant effect on the measured glycogen content of cardiac muscle.[28] When beating hearts were excised by severing the great vessels in a single cut, it was observed that contraction continued while blocks were taken and for a brief period after immersion of the tissue in fixative. Although this was done as quickly as possible, there was often significant generalized reduction of glycogen in the normal areas of the myocardium. Uncontrolled glycogen losses from normal areas made very difficult the interpretation of the loss noted in areas subject to very short periods of ischemia. The oxygen usage of the empty, contracting, of the arrested, or of the fibrillating, perfused heart is about 20 to 30 per cent of its consumption when beating and working.[29-31] Metabolic utilization of labile glycogen is more rapid in the contracting anoxic heart which is doing work than in the nonworking anoxic heart.[22]

Artifactual glycogen breakdown was markedly reduced by the rapid excision and fixation of hearts from animals in which cardiac arrest was produced by injection of potassium or calcium and in which oxygenation was maintained by positive pressure respiration. The mere loss of cardiac glycogen in no way indicates that an irreversible change has occurred or will occur. A functioning frog heart preparation, devoid of glycogen, has been described.[32] It has been well documented [24,25,33-35] that temporary occlusion of coronary arteries in the dog for periods less than 20 to 30 minutes does not result in permanent myocardial damage. Electron microscopic observation of tissue from this series of rabbits suggests that the initial morphologic evidences of irreversible alteration may be the loss of ability of the ischemic fiber to contract when placed in

osmium tetroxide, and the swelling of the mitochondria. These changes occur 20 to 30 minutes after coronary occlusion.[1]

The presence of glycogen in the myocardial fiber does not indicate the viability of the cell. Complete autolytic degradation of myocardial glycogen after death may require 4 to 6 hours at 37° C. and much longer at 4° C.[36-38] It might be expected that hearts which suffer sudden cardiac arrest while well oxygenated and well supplied with glycogen might retain much of it for several hours after death. Hearts which continue to beat or fibrillate after cessation of respiration or oxygenation of the blood might be expected to be almost totally depleted of glycogen even before death. In some instances it has been possible for us to demonstrate in human subjects sharply demarcated areas of glycogen loss in hearts which showed no other morphologic alterations as sequels to recent coronary occlusion. The absence of glycogen as an indicator of early myocardial ischemia might be of greater diagnostic value to the pathologist if necropsies were performed immediately after sudden death from coronary occlusion. Pathologists in forensic practice may have such an opportunity.

Knowledge of early changes caused by ischemia has stimulated studies, now in progress, to investigate the reversibility of these lesions and the conditions under which irreversible alterations can be prevented or delayed. Such information may be important to an understanding of cardiac physiology and to the surgeon utilizing extracorporeal circulation and cardiac arrest.

The nature of the PAS-positive substance which appears in ischemic muscle fibers is not known.[26,27] The earlier recognition of this material in tissues exposed to protein-precipitant fixatives suggests that the chemical compounds which provide the vicinal reactive groups for the PAS reaction may be relatively water soluble substances which, when present only in small quantity, can be retained in the sections by chemical precipitation and binding but not by dehydration alone.

A similar mechanism can be suggested for the unusual staining reactions noted in the Z bands. The labile or nonprotein-bound glycogen has predominantly a perinuclear distribution and undergoes rapid alterations with anoxia or other conditions favoring glycogenolysis. The cellular localization of the glycogen portion designated as desmo-, stable, or protein-bound has not been demonstrated. The techniques for chemical separation of extractable and residual glycogen[28] depend on the insolubility of residual glycogen after grinding and fixation of the tissue with trichloroacetic acid.

It has been suggested[39] that much of the glycogen in human muscle is resistant to diastase digestion and that this resistant fraction is present

in the cross striations. The present findings tend to confirm and extend these observations. It may be suggested that the stable fraction of glycogen is closely related to proteins located along the Z bands. Following the mild denaturation and dehydration produced by alcohol or acetone, this glycogen may be removed by diastase digestion. However, after exposure to protein-precipitant fixatives, the glycogen is rendered resistant to digestion with diastase.

Morphologic and histochemical changes observed in these rabbits at longer intervals after coronary ligation confirm the findings noted by many investigators in man and in the dog. Evidences of inflammatory reaction seem to develop more rapidly than they do in larger animals. This may be explained in part by the high metabolic rate, by the relatively large reactive perimeter present about small lesions, by the necessity of passing the suture needle and thread into the myocardium, and by the use of nonsterile operative techniques.

The appearance of fat 12 hours after ligation corresponds in general to observations described in man[37] and in the dog.[25] Fatty degeneration has been reported in the dog as early as 4 hours after coronary ligation.[40] Unlike the situation in man, where fatty degeneration often appears before other evidences of infarction can be detected in hematoxylin and eosin stained preparations, fatty degeneration in the rabbit does not occur until long after the infarct can be detected by conventional stains.

A high incidence of myocarditis of unknown etiology has been reported in domestic rabbits.[41] There is evidence to suggest that a virus is responsible.[42] It is disturbing to find spontaneous myocardial disease in an experiment designed to produce myocardial lesions. It is felt, however, that the spontaneous and experimental lesions are sufficiently different in their distribution, pattern, size, age, and histologic characteristics that they should not be confused. Most of the animals with severe myocarditis died during or shortly after the operative procedure and thus were excluded from this study. The number of animals in each time group was sufficiently large that two or more hearts without evidence of myocarditis were available for study.

Summary and Conclusions

A technique has been developed for the experimental production of uniform and sharply delimited myocardial infarction in the rabbit. Morphologic alterations following experimental coronary arterial ligation in the rabbit are similar to those previously described in the dog. The earliest lesion in evidence of myocardial ischemia is loss of the labile fraction of glycogen. This may be detected histochemically within one minute after ligation of a coronary artery. Artifactual loss of cardiac

glycogen may occur during the period of animal sacrifice and preparation of the tissue for fixation. This may be decreased by rapid induction of cardiac arrest. The "desmo" component of cardiac glycogen is located along the Z bands. It is rendered resistant to diastase digestion following tissue fixation with protein-precipitant fixatives.

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[*Illustrations follow*]

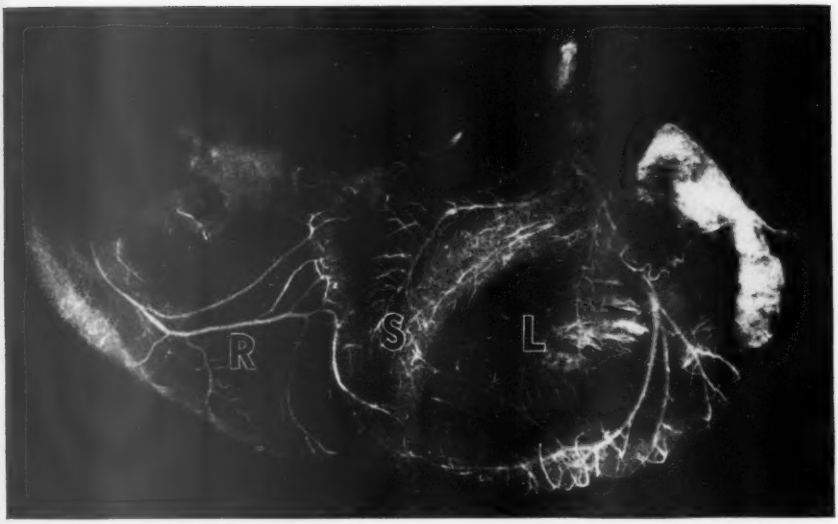
Legends for Figures

Fig. 1. This roentgenogram of the coronary arteries in the rabbit heart was prepared by intra-aortic retrograde injection of a barium sulfate-gelatin mixture. The heart was opened by a flat, unrolling technique. L, R and S refer to left ventricle, right ventricle, and septum, respectively.

Fig. 2. Vinyl acetate cast of rabbit coronary arteries and left ventricle. This cast was produced by retrograde intra-aortic injection of vinyl acetate and subsequent digestion of the tissue with potassium hydroxide. The cast demonstrates vessels, aortic semilunar valves and papillary muscles. Note the abrupt termination (arrow) of a branch of the left coronary artery produced by coronary ligation.

Fig. 3. Photomicrograph showing extensive myocarditis in a rabbit which died spontaneously. Myocardial fibers are separated by edematous spaces containing cardiac histiocytes. Hematoxylin and eosin stain. × 640.

Fig. 4. Detail in a focus of myocarditis from a rabbit which died 55 minutes after coronary artery ligation. Hematoxylin and eosin stain. × 1700.



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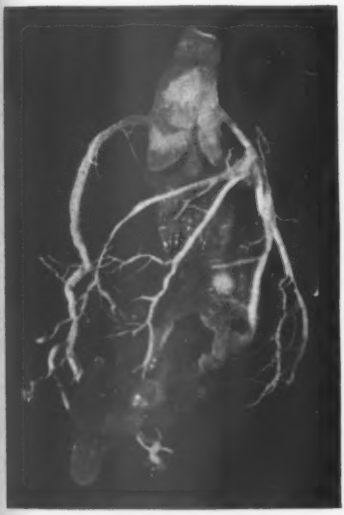

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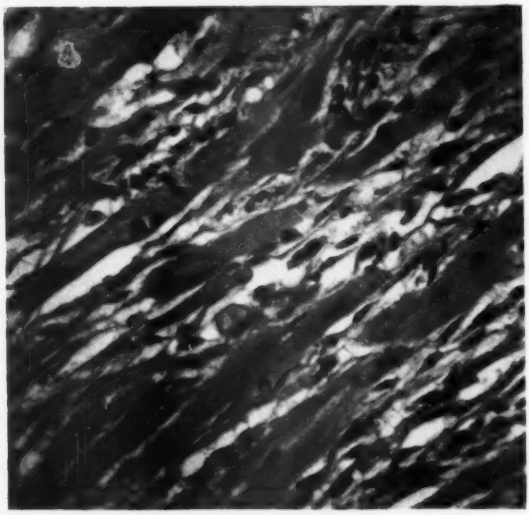

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Figures 5 to 7 are photomicrographs showing junctional zones between normal and ischemic myocardium at various intervals after coronary artery ligation. All animals were sacrificed by excision of the beating heart which was fixed in absolute alcohol and stained with periodic-acid-Schiff (PAS) stain. Ischemic areas are to the left and normal areas to the right.

FIG. 5. Five minutes after ligation. Note glycogen flight. Much glycogen is lost from the normal area by this technique, and recognition of early changes is difficult. × 180.

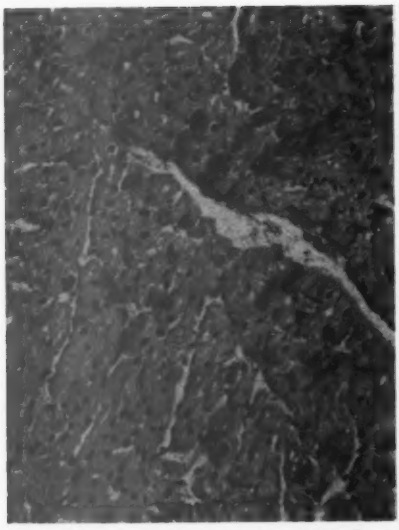
FIG. 6. Fifteen minutes after ligation. There is interstitial edema at the junction zone and retention of glycogen beneath the endocardium at the upper left. × 39.

FIG. 7. One hour after ligation. Note the preservation of glycogen in the subendocardium and about thebesian veins. × 18.

FIG. 8. Demarcation between ischemic and normal cardiac muscle produced by loss of glycogen within one minute after coronary ligation. The ischemic area is on the left. The heart was arrested by intravenous injection of calcium chloride and was fixed in absolute alcohol containing one per cent mercuric chloride. PAS stain. × 160.

FIG. 9. Glycogen is abundantly present in normal cardiac muscle following cardiac arrest with potassium chloride. Note the predominant perinuclear distribution. Freeze-dried. PAS stain. × 1700.

FIG. 10. Cryostat section of dog heart 5 minutes after ligation of the left circumflex coronary artery and induction of cardiac arrest with potassium citrate. A fresh frozen section was cut in the cryostat and post-fixed in one per cent mercuric chloride in absolute alcohol. This technique prevents the glycogen flight and demonstrates perinuclear distribution of glycogen in the normal area. PAS stain; green filter. × 40.



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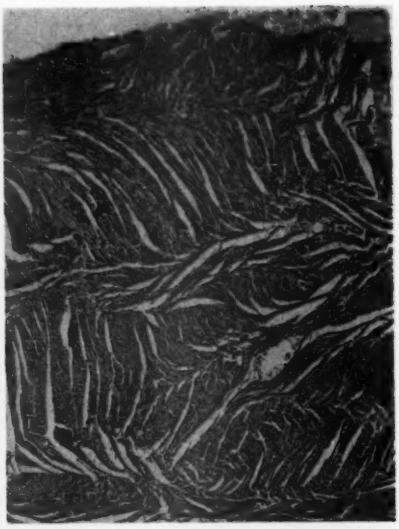

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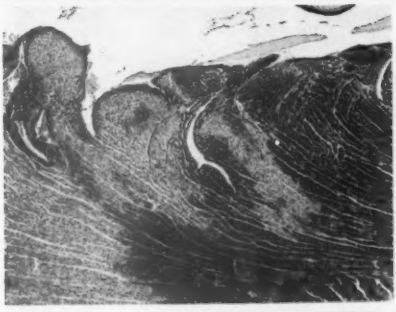

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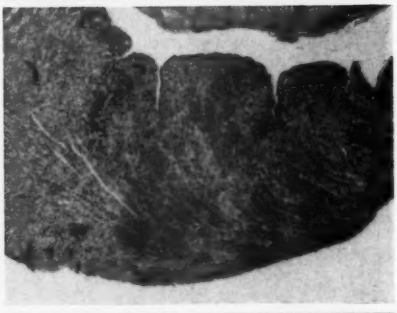

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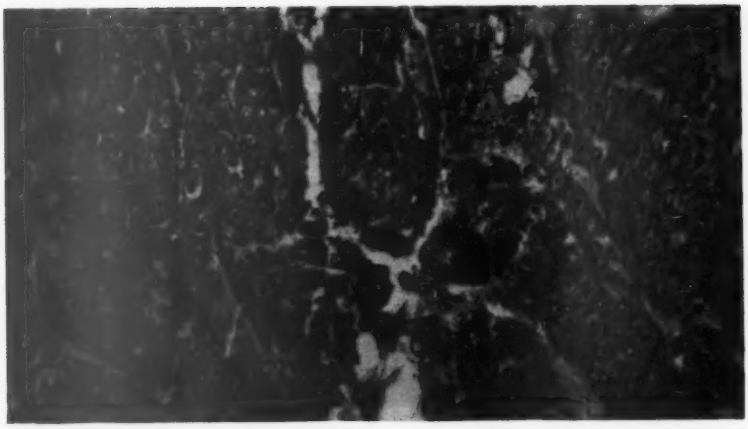
FIG. 11. After two hours of ischemia, the muscle to the left shows a diffuse cytoplasmic reaction with PAS stain after diastase digestion. This technique has removed labile glycogen from normal muscle at the right. Picric alcohol-formalin fixation; PAS stain. × 18.

Figures 12 and 13 demonstrate the effect of fixation on the ability of diastase to remove glycogen from the Z bands of cardiac muscle.

FIG. 12. The Z bands were magenta. Fixed in neutral buffered formalin; diastase digestion; PAS and hematoxylin stain; green filter. × 1700.

FIG. 13. Although the Z bands did not color, the sarcolemma and capillary basement membranes still react with the stain. Alcohol fixation; PAS and hematoxylin stain; green filter. × 1700.



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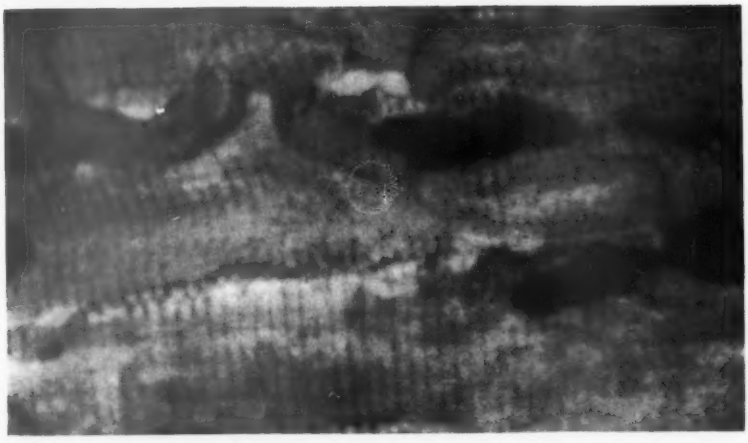

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THE STRUCTURE AND SIGNIFICANCE OF PULMONARY PLEXIFORM STRUCTURES

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Curious malformations fill portions of the pulmonary arterial tree in certain persons with advanced pulmonary arterial hypertension.[1-6] These formations bear a resemblance to vascular glomuses and to recanalized thrombi and have been termed plexiform structures. It has been suggested that these structures are responsible for the high pulmonary vascular resistance seen in many cases of patent ductus arteriosus, interventricular septal defect, and interatrial septal defect. Despite widespread interest, the exact structure and significance of these alterations has remained in dispute.[2,4-6]

In this study, plexiform structures in patients with patent ductus arteriosus, interatrial septal defect, and primary pulmonary hypertension were reconstructed from serial microscopic sections. In addition, vascular dimensions throughout these structures were determined by direct measurement. The observations suggest that the plexiform structures were responsible for the high pulmonary vascular resistance found in these individuals during life. The rather uniform morphologic pattern of the structures, independent as it is of the underlying disease, suggests a common denominator in their pathogenesis.

CLINICAL DATA

Historical and laboratory data are detailed in Tables I and II. Three patients each had a patent ductus arteriosus, two had large interatrial septal defects of the heart, and the sixth, idiopathic pulmonary hypertension. Many clinical features were common to the group. All complained primarily of long-standing weakness, fatigue and progressive exertional dyspnea. Several experienced angina pectoris, orthopnea and periodic hemoptysis. On physical examination, all had an accentuated pulmonic second sound. All were cyanotic at rest. Two of the patients with patent ductus arteriosus had continuous murmurs to the left of the sternum, and one had a diastolic murmur in this region. A coarse systolic murmur to the left of the sternum was heard in one patient with interatrial septal defect whereas in the other, both systolic and diastolic

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TABLE I

CLINICAL AND NECROPSY OBSERVATIONS

	Patent ductus arteriosus			Interatrial septal defect		Primary pulmonary hypertension
	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Symptoms and signs						
Age (yr.) and sex	31 F	33 F	32 F	37 F	43 F	31 M
Dyspnea	1+	2+	2+	2+	2+	2+
Fatigue	1+	1+	1+	1+	2+	1+
Angina pectoris	1+	1+	0	1+	0	0
Orthopnea	0	0	1+	1+	1+	0
Hemoptysis	1+	1+	0	0	0	1+
Duration (yr.)	6	4	9	2	22	9
Physical examination						
Accentuation of P_2	2+	2+	2+	2+	2+	2+
Right heart failure	1+	2+	2+	2+	2+	2+
Cardiac murmurs	Diastolic to left of sternum	Systolic in pulmonic area; soft diastolic to left of sternum	To-and-fro murmur in 2nd left interspace	Coarse systolic to left of sternum	Loud systolic and diastolic at apex	None
Laboratory						
Hematocrit	55	71	62	47	56	54
Thoracic roentgenogram						
Cardiac enlargement	2+	2+	2+	3+	3+	2+
Enlarged pulmonary artery	+	+	+	+	+	+
Electrocardiogram						
Right ventricular hypertrophy	+	+	+			+
Right bundle branch block				+	+	
Necropsy	*	†				
Gross thrombotic material in pulmonary arteries	0	0	0	+	0	+
Complexity of plexiform structures	3+	3+	2+	2+	1+	3+
Weight of heart (gm.)	355		450	540	600	550
Thickness						
Right ventricle (mm.)	1.2		1.3	1	1	1.5
Left ventricle (mm.)	1.6		0.8	1	1	1.3
Dimensions of cardiac malformation	Diam., 1 cm.; length, 0.75 cm.	Diam., 3 cm.; length, 2.5 cm.	Diam., 1.2 cm.; length, 0.8 cm.	Diam. of defect, 2.5 cm.	Diam. of defect, 0.8 cm.	No malformations

Key: 0 = absent; 1 + = mild; 2 + = moderate; 3 + = severe

*Limited to thorax. † No organs removed.

Key: 0 = absent; 1 + = mild; 2 + = moderate; 3 + = severe

*Limited to thorax. † No organs removed.

TABLE II

CARDIAC CATHETERIZATION DATA

	Patent ductus arteriosus			Interatrial septal defect		Primary pulmonary hypertension
	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Pressures (mm. of Hg)						
Pulmonary arterial	103/68	126/82		95/47	110/43	107/61
Mean pulmonary arterial		96		67	60	79
Mean right atrial		4	2	10	9.5	5
Mean left atrial					8.5	
Brachial artery	102/64	123/83	100/60	125/90	125/75	122/70
Oxygen saturation						
Superior vena cava		62%		48%	58%	49%
Right atrium	51%	61%		47%	75%	49%
Right ventricle	57%	65%		49%	76%	49%
Pulmonary artery	68%	67%		54%	78%	49%
Pulmonary vein					93%	
Brachial artery	84%	81%		74%	84%	90%
Comments	Predominant left to right ductus shunt but some right to left shunt also	Predominant right to left ductus shunt but some left to right shunt also	Catheterization unsuccessful; cyanosis suggests right to left shunt	Right to left atrial shunt	Both left to right and right to left atrial shunts	

murmurs were detected. Electrocardiograms and roentgen studies gave evidence of right ventricular preponderance in all.

Pulmonary arterial pressures were markedly elevated in all cases and in two instances matched those in the brachial arteries (Table II). Some degree of right to left shunt was revealed by oxygen saturation studies in the patients with cardiac malformations. In those cases in which it was patent, the ductus arteriosus at necropsy was relatively short and wide. When present, interatrial septal defects were large (Table I).

The patient with primary pulmonary hypertension had an unusual history. He participated in decompression experiments 9 years before death and had several episodes of decompression sickness. Soon afterwards, he developed increasing dyspnea and experienced intermittent hemoptysis. This was progressive until death. Late in the illness, angiograms revealed delayed emptying of enlarged pulmonary arteries while giving no evidence of intracardiac or intrapulmonary shunts. Brachial artery oxyhemoglobin saturation was 90 per cent. At necropsy no cardiac malformations were found. There were no abnormal communications between the various intrapulmonary vessels, and there was no primary parenchymal disorder of the lungs which might have narrowed the vascular bed.

TABLE III

ANALYSIS OF 10 WELL DEVELOPED PLEXIFORM STRUCTURES
(CASE 1)
AVERAGE CROSS-SECTIONAL AREA

	Area in square μ				
	Lumen	Intima	Media	Total area of vessel	Length (μ)
Artery immediately proximal to the plexiform structure	2,374	2,419	1,294	6,087	
Afferent channel of the plexiform structure	637	4,826	None	5,463	153
Main plexiform structure	907	31,086	None	31,993	800
Artery or arteries immediately distal to the plexiform structure	6,848	885	30	7,763	

METHODS

To insure that structures investigated were representative, tissue was obtained from all lobes of each lung examined. Each block of tissue was serially sectioned at 6 μ. Sections were stained with hematoxylin and eosin or van Gieson's stains and counterstained for elastic tissue. The plexiform structures were easily identified in almost every section and were analyzed by two methods. First, individual plexiform structures

and the vessels proximal and distal to them were reconstructed from camera lucida drawings of serial sections. Brewer's method[7] was used, except that overlapping vessels were separated to facilitate visualization in two-dimensional models (Text-figs. 1 and 2, and Figs. 1 to 3). Secondly, the component parts of 10 structures and associated vessels were measured (Table III). The latter method made it possible to compare the sizes of vessel lumens, media, and intima at various levels. The serial section technique permitted accurate separation of bronchial and pulmonary vascular systems. Prior injection with gelatin preparations[8] did not significantly facilitate identification at microscopic levels.

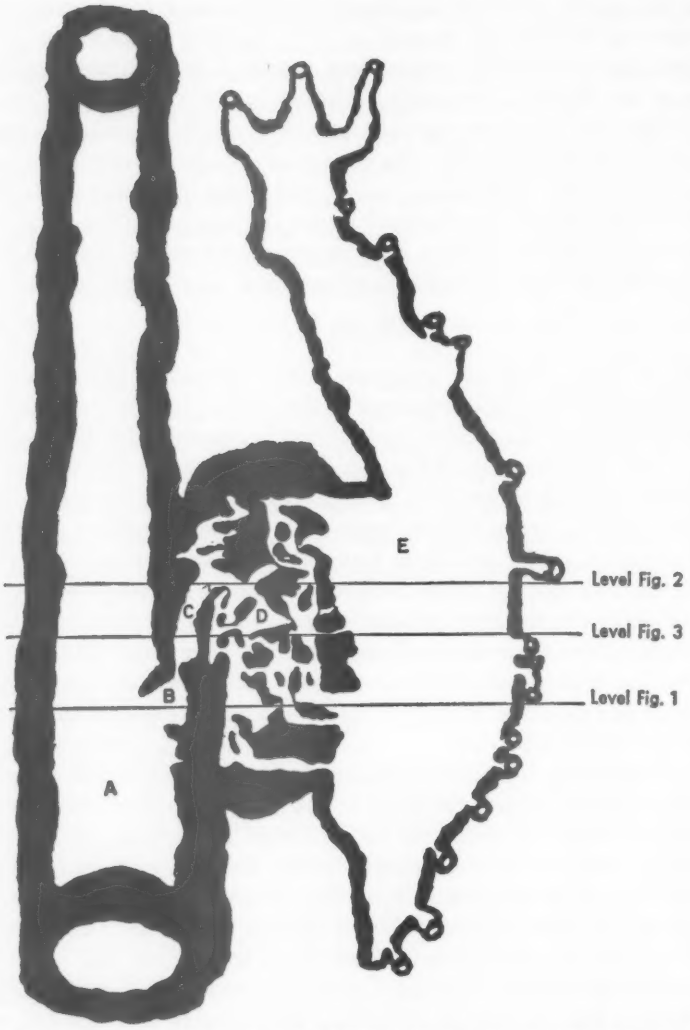
Results

In all cases, plexiform structures were centered in the pulmonary muscular arteries. For convenience of description, the muscular arterial systems in these cases can be divided into 3 segments: (a) those proximal to the plexiform structures; (b) those involved by the structures; and (c) those distal to them. The proximal segments connected with elastic arteries, and the distal segments with the pulmonary arterioles and capillaries. The structural features of the various segments were sufficiently alike in the 6 cases to allow a composite anatomic description.

Alterations in the arterial segments proximal to the plexiform structures resembled those previously reported.[2,3,9] They consisted of intimal connective tissue proliferation and medial smooth muscle hypertrophy (Figs. 1 and 5). These alterations resulted in variable degrees of narrowing of the arterial lumens.

The plexiform structures had a characteristic pattern, and a typical one is reconstructed in Text-figure 1. Just proximal to them, the involved arterial channel was invariably much reduced by sclerosis. This narrow, tortuous channel was usually single, its wall formed by connective tissue which was often hyalinized. Since this channel led into the plexiform structure, we termed it the "afferent channel" (Text-fig. 1 and Figs. 2 and 5). The afferent channels (Text-fig. 1, area C) and the plexiform structures themselves (Text-fig. 1, area D) were located most frequently in arteries near a point of branching. By referring to Table III, one may see that the average "afferent channel" had a lumen area only 27 per cent of that of the immediately proximal arterial segment. The average "afferent channel" was quite long, measuring 153 μ in length. At its distal end, the "afferent channel" opened into the main mass of plexiform channels (Text-fig. 1 and Fig. 2).

The main plexiform structure resembled the "afferent channel" except that it was composed of many narrow channels instead of one or two. Like the "afferent channel," these were embedded in a mass of



TEXT-FIGURE 1. Reconstruction of a plexiform structure from serial sections (case 1). Arterial walls are shaded. (A) is a muscular artery proximal to the plexiform structure. Its wall is thickened by medial hypertrophy and intimal fibrosis. Just beyond (B) a branching artery is markedly narrowed by fibrosis to form a narrow "afferent channel" (C). The latter channel joins the narrow, anastomosing plexiform channels (D). Some of the plexiform channels end blindly, but others coalesce distally into another segment of the same arterial channel (E). Labeled lines show levels at which sections in Figures 1 to 3 were taken.

connective tissue. Within the mass, many of the channels ran parallel to one another while others were serpiginous and interconnected. A few

ended blindly, and a small number contained organizing thrombi. Like the lumens of the "afferent channel," those of the plexiform structures varied from 4 to 18 μ in diameter. As shown in Table III, the total cross-sectional area of the lumens averaged 37 per cent of that of the proximal

TEXT-FIGURE 2. A typical plexiform structure is reconstructed from serial sections. The solid lines outline the vascular lumens. In this instance, plexiform channels fill several nearby branches of an artery. (Case 6).

arterial segment. The plexiform structures were long, extending for an average of 800 μ from "afferent channel" to distal muscular artery. A sufficient number of the former elastic membranes remained to indicate that the majority of the channels had formed within the pre-existing artery (Figs. 4 and 5). However, a few channels passed between and others outside the membranes while interplexiform channels were occasionally seen. Some of the latter may have represented normal branches of the parent artery (Text-fig. 2 and Fig. 4).

The plexiform channels eventually coalesced to form a variable number of dilated vessels, the distal segments of the muscular arterial tree (Text-fig. 1 and Figs. 3 and 5). These arteries had thin muscular coats, the remainder of their walls being normal. They in turn joined normal arterioles and capillaries. No abnormal communications were found be-

tween the pulmonary arteries and veins or any part of the bronchial vascular system.

The exact position of the plexiform structures in the muscular arterial system varied somewhat from case to case. In the cases of patent ductus arteriosus, the majority appeared in muscular arteries from 200 to 400 μ in outside diameter. In interatrial septal defect, they were also found in arteries smaller than 200 μ. In the case of primary pulmonary hypertension, muscular arteries of all sizes were affected.

In all cases, an attempt was made to determine what proportion of arteries was involved by plexiform structures. Most of the muscular arterial channels were found to have a single interposed plexiform structure at some point. However, even in the most advanced case, not quite every channel was so affected. In case 1, 80 channels were traced, and only 3 failed to show a plexiform structure. In these 3, much larger arterial lumens narrowed to less than 20 μ at some point. Arteries beyond these narrow segments resembled those seen distally to the plexiform structures.

Discussion

Plexiform vascular structures were found to be common in the pulmonary arteries of 6 persons with advanced pulmonary hypertension. Five had cardiac malformation permitting large shunts of blood between the two circulations, and the sixth had idiopathic pulmonary hypertension. In these cases, plexiform structures were found interposed in almost every arterial channel examined. The plexiform and afferent channels were found to be the narrowest segments of the pulmonary arterial tree. Most had a lumen size similar to that of a systemic arteriole. The channels were also presumably rigid since their walls were formed by fibrous tissue. Lacking an alternate route in the lesser circulation, blood must have passed through them to have reached the distal arteries and pulmonary capillaries. Reference to Poiseuille's law * suggests that they imparted a high resistance to the pulmonary circulation.

Patients with pulmonary plexiform structures have been reported to have such a high, fixed, pulmonary vascular resistance that cardiac output is reduced.[2] This appeared to be true in the current cases. The weakness, fatigue and lethargy experienced by these patients could probably have been attributed to inadequate cardiac output. Low cardiac output may also have contributed to the angina pectoris experienced by 3 patients since they had only slight coronary arteriosclerosis. High pulmonary arterial pressures were also evidence of high resistance in the lesser

* For a given rate of flow, the resistance to flow is inversely related to the fourth power of the radius of the vessel's lumen.

circulation. Resistance approached that of the systemic circulation in the cases with cardiac malformations. This was evidenced by reversal of earlier left to right shunts. The degree of shunt reversal in each case was roughly proportional to the number of plexiform structures present.

The oxyhemoglobin unsaturation and secondary polycythemia observed might be used as an argument for pulmonary arteriovenous shunts. The argument for such shunts has recently been revived.[4-6] However, the evidence suggests that the unsaturation reflected the cardiac shunts. The full saturation of pulmonary venous blood in one case was additional evidence that significant pulmonary arteriovenous shunting did not exist (Table II). In other cases, injection of the pulmonary arterial tree at necropsy and serial section examination demonstrated no direct communications between the arterial and venous systems. There is no ready explanation for the slight arterial unsaturation in the case of idiopathic pulmonary hypertension.

Three factors may be significant in the pathogenesis of plexiform structures. The structures may represent: (a) recanalized thrombi or emboli[10]; (b) a reactive fibrosis associated with high pressure, high flow and turbulence[2]; or (c) reactive fibrosis following gas emboli to the lesser circulation.[11] Insufficient evidence is available to ascribe the primary role to any of these factors. However, there appears to be more direct evidence to support the role of thrombi than to support the other hypotheses.

It is well established that organization of thrombi or emboli in the pulmonary arteries may lead to intimal fibrosis. This has often been reported in primary pulmonary hypertension.[1] It seems likely that thrombi or emboli had an important role in the current cases. One of the cases of interatrial septal defect, as well as the example of primary pulmonary hypertension, had pulmonary arterial thrombi. Thrombi were also found in channels of the plexiform structures in all of the cases. Other authors have found such thrombi to be rare. This has led them to attribute the development of the plexiform structures to a reactive fibrosis associated with high pressure, flow and turbulence.[2] They have likened the fibrotic plexiform lesions to the "jet fibrosis" found in the pulmonary artery opposite a patent ductus or in the vessels associated with a congenital arteriovenous aneurysm.[2] It must be remembered, however, that while it takes weeks for thrombus substance to organize, it usually takes years for the complex plexiform structures to develop. This probably explains why random sections taken at any single time in this long process may demonstrate so little recent thrombotic substance.

The possible role of gas emboli in the pathogenesis of the structures

is less well established. Experimentally, gas emboli may lead to intimal fibrosis.[11] Such emboli may have initiated the vascular changes in the patient with primary pulmonary hypertension. They may have been associated with the "bends" which he experienced when undergoing decompression experiments.

Summary

This report concerns the structure and significance of plexiform structures, lesions in the pulmonary arterial tree which accompany some types of cardiac malformations and primary pulmonary hypertension. The structures are advanced lesions which interpose a significant obstruction in the pulmonary arteries. Some of the vascular alterations were quantitated in an attempt to correlate them with observed changes in pulmonary vascular resistance. The region of maximum restriction to blood flow was often found to be the arterial segment just proximal to the main plexiform mass.

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[*Illustrations follow*]

Legends for Figures

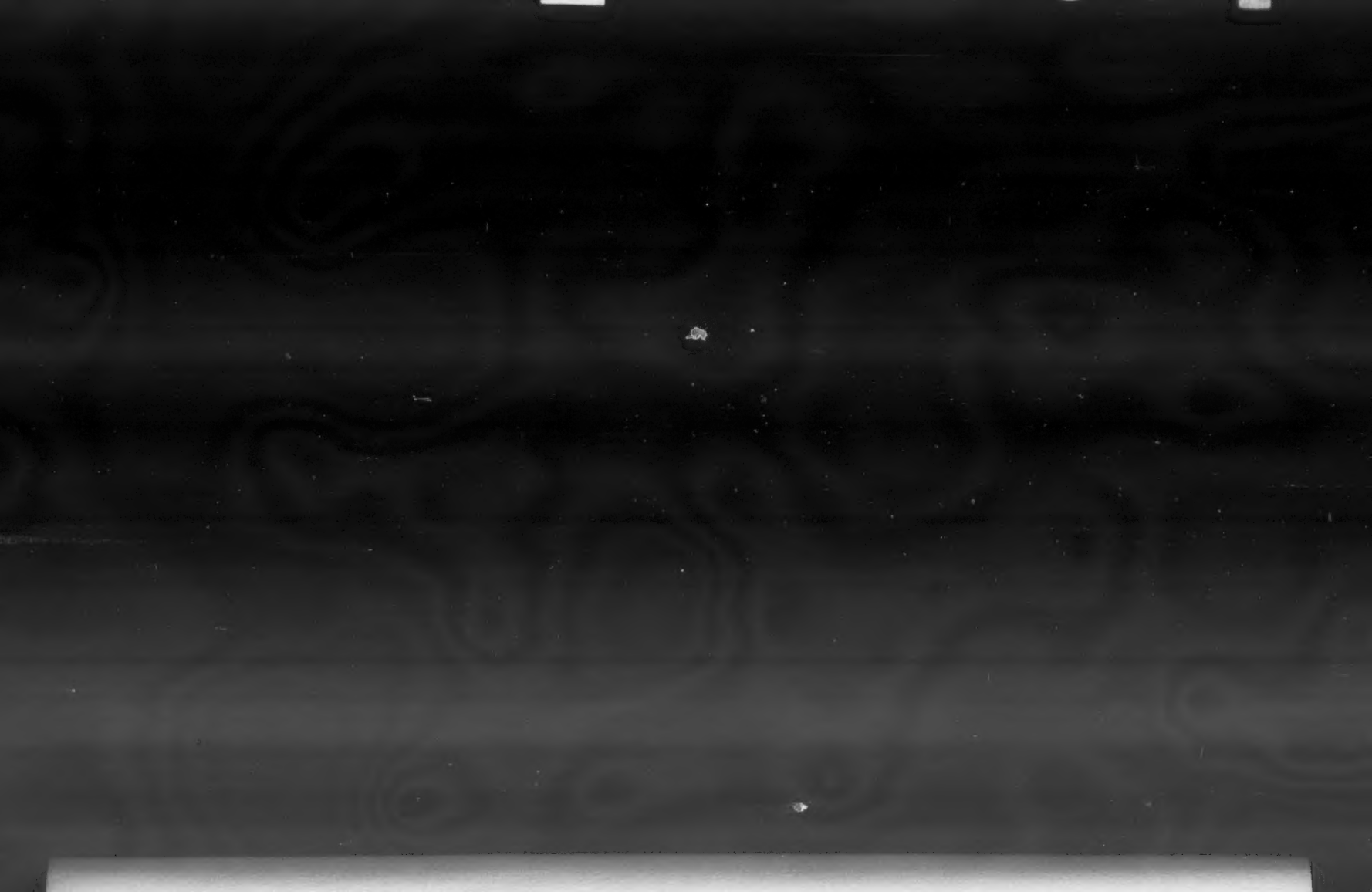
Fig. 1. Section through the plexiform structure reconstructed in Text-figure 1. In the center a muscular artery branches into the vessel proximal to the plexiform structure. Plexiform channels are seen above. Hematoxylin and eosin stain. × 120.

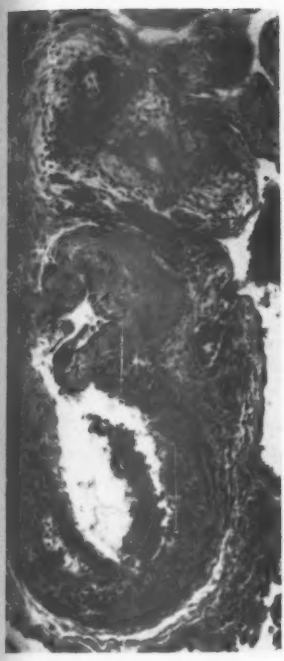
Fig. 2. Section through the plexiform structure reconstructed in Text-figure 1. At a point just above center, the "afferent channel" joins the first plexiform channel. The main mass of anastomosing channels is seen above and below. Hematoxylin and eosin stain. × 120.

Fig. 3. Section through the plexiform structure reconstructed in Text-figure 1. The narrow "afferent channel" is seen to the left of center. Just to the right, plexiform channels are coalescing into another segment of the arterial channel, a dilated muscular artery. This distal dilated artery can be seen along the right edge of Figures 1 and 2. Hematoxylin and eosin stain. × 120.

Fig. 4. Section through the plexiform structure reconstructed in Text-figure 2. Hematoxylin and eosin and Verhoeff's stain. × 120.

Fig. 5. A plexiform structure from case 2. Below, the proximal arterial segment narrows to form the "afferent channel." In the center are seen the anastomosing channels of the plexiform structure. At the upper left, plexiform channels have coalesced to join the dilated, distal artery. Hematoxylin and eosin and Verhoeff's stain. × 120.





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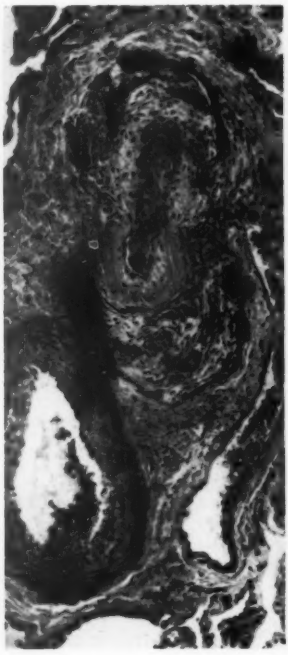

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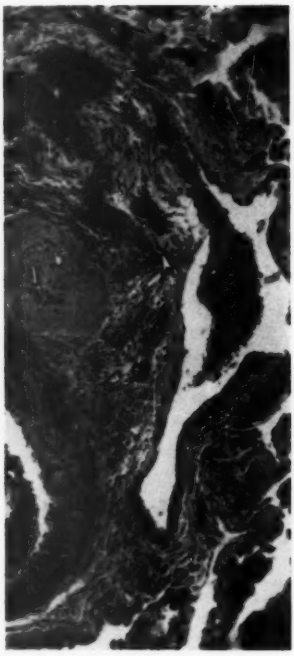

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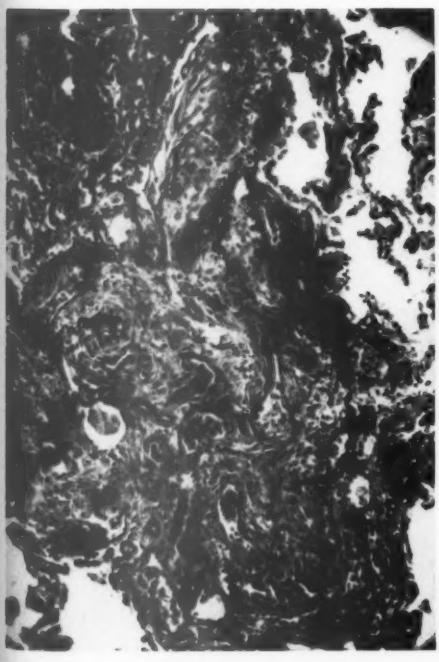

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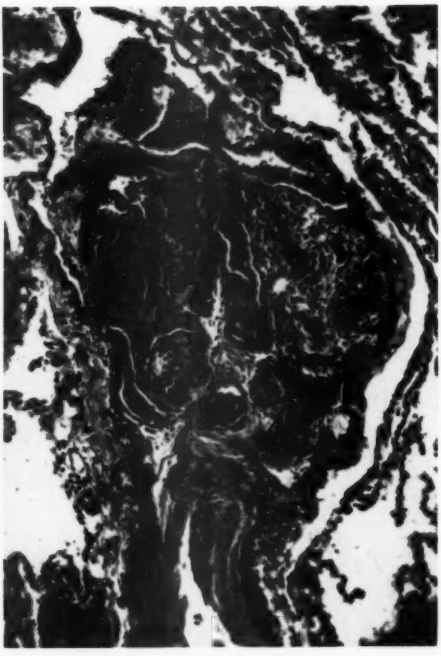

5

CHRONIC GLOMERULONEPHRITIS

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The essential feature of chronic azotemic glomerulonephritis is obsolescence of the glomeruli. This is accompanied by tubular atrophy, interstitial fibrosis and inflammation, and contraction of the kidneys. The alterations are progressive in nature. They follow those of subacute glomerulonephritis [1,2] and terminate in complete dissolution and disappearance of glomeruli [3,4] with scarring of the parenchyma. The development of these changes and the structure of the obsolete glomeruli have been investigated by means of the thin section technique.[5]

MATERIAL AND METHODS

Twenty-five cases of chronic glomerulonephritis were used in this study. These came in the main from the files of the Mount Sinai Hospital, New York City, and the Barnert Memorial Hospital, Paterson, New Jersey. Case 24 was obtained from Fordham Hospital through the courtesy of Dr. L. Millman, and case 25 from the Lebanon Hospital through the courtesy of Dr. J. Ehrlich. The methods were identical with those used in previous studies of acute and subacute glomerulonephritis.[2,5]

RESULTS

Clinical data and anatomic features are summarized in Tables I and II. The histologic lesion of chronic glomerulonephritis is characterized by a large number of obsolete glomeruli which in our cases varied between 40 and 90 per cent, averaging about 65 per cent. Obsolete glomeruli are those which have lost their structure and function completely; they are usually the seat of fibrosis or hyalinization. Among these, some were in the process of dissolution. A number of obsolescent glomeruli showed partial loss of structure, while a few were fairly well preserved and exhibited the typical alterations of subacute glomerulonephritis. In the obsolescent glomeruli the lobular architecture was often exaggerated, probably because of increased compactness and straightening of in-

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TABLE I

CLINICAL AND GROSS PATHOLOGIC FEATURES IN 25 CASES OF CHRONIC GLOMERULONEPHRITIS

Case no.	Sex	Age	Duration of illness	Blood pressure	Edema	Albuminuria	BUN (mg.)	Blood cholesterol (mg.)	A/G ratio (gm. per 100 cc.)	Kidneys: combined wt. (gm.)	Remarks
1	M	32	15 yr.	220/130	0	4+	102	211	4.4/1.8	190	
2	M	6½	3 yr.	168/140	+	4+	92	960	2.5/3.1	120	Nephrotic syndrome for 2 yr. at onset
3	M	14	2 yr.	165/120	4+	3+	122–250	ND	3.2/2.2	200	Acute glomerulonephritis, history
4	M	54	3 yr.	155/95	0	3+	108–136	ND	3.1/4.2	230	
5	M	57	2 yr.	140/80	0	3+	205	ND	ND	200	Nephrotic syndrome, history
6	M	8	3 yr.	125/90	2+	4+	156	ND	ND	60	Nephrotic syndrome 6 mo. before death: A/G = 2.7/2.9; cholesterol, 720; acute exacerbation
7	F	13	6 yr.	160/100	0	3+	138	380	2.5/2.6	90	Nephrotic syndrome, history
8	M	24	? 2½ mo.	150/120	0	3+	170	ND	3.2/3.3	255	Acute exacerbation
9	F	46	23 yr.	210/130	1+	2+	111	340	ND	95	
10	M	32	14 yr.	200/120	0	4+	170–232	ND	ND	250	Sympathectomy for hypertension
11	M	27	9 yr.	180/118	0	4+	94	ND	3.5/3.1	250	
12	M	38	8 mo.	210/140	0	4+	210	ND	ND	250	
13	M	64	2+ yr.	170/90	0	3+	161–232	328	4.0/3.7	140	
14	M	40	8 yr.	152/88	0	4+	164	170	3.3/3.4	190	
15	F	16	6–10 yr.	185/115	0	4+	240	250	3.6/3.1	115	
16	M	74	ND	140/80	0	3+	186	236	2.8/3.3	230	Acute exacerbation
17	M	36	4 yr.	196/125	0	4+	124–220	384	2.8/3.2	170	Acute glomerulonephritis, history
18	M	20	1½ yr.	210/120	3+	3+	96–218	242	2.3/3.5	165	Nephrotic syndrome, history
19	M	28	3½ yr.	220/152	0	4+	100	ND	3.8/3.0	190	Left kidney absent (congenital)
20	M	52	10 yr.	150/90	0	3+	178–372	ND	3.9/3.0	180	
21	M	53	2½ yr.	220/110	2+	4+	140	500	ND	245	Nephrotic syndrome
22	M	29	22 yr.	220/140	0	4+	330	ND	3.7/1.3	ND	
23	M	7	4 yr.	110/70	3+	4+	190(NPN)	489	1.4/1.9	ND	Nephrotic syndrome, history
24	M	10	ND	200/130	2+	4+	23	ND	ND	50	
25	M	20	2+ yr.	150/104	0	4+	208	288	2.5/2.6	ND	

Key: BUN = Blood urea nitrogen. A/G ratio = Albumin to globulin ratio. ND = No data. NPN = Nonprotein nitrogen.

Table II

MICROSCOPIC FEATURES IN 25 CASES OF CHRONIC GLOMERULONEPHRITIS

Case no.	No. of obsolete glomeruli per hundred		Intercapillary space		Capillary wall				Crescents	Blood vessels	
	Total	Hyalin	Fibrosis	Hyalin	Thickening	Splitting	Hyaline deposit	Membranous transf.		Arterio-sclerosis	Arteriolo-sclerosis
1	75	15	+	++	o	++	+	o	o	++	++
2	60	10	++	+	+	++	+	o	++	o	++ necrosis
3	50	40	+	+++	o	++	+	o	+	o	++
4	60	10	+++	++	o	+	+	o	+	++	+
5	50	5	+	o	o	++	o	++	+	+++	+
6	60	10	++	o	+	++	o	o	+	o	+ occ. necrosis
7	65	15	+	+	+	o	o	o	++	o	+
8	65	60	++	++	o	+	o	o	++	o	o
9	85	10	++	++	o	++	+	o	+	+++	++
10	85	25	++	++	o	+	o	o	o	+++	+++
11	75	65	++	+++	+	+	o	o	+	+	+++ necrosis
12	50	10	++	+	++	++	+	o	+	+	+++ necrosis
13	90	10	+	+	o	++	o	o	++	++	++
14	75	30	+	++	o	o	o	o	++	+	++
15	65	55	++	+++	o	++	+	o	+	+	++
16	40	5	+	+	+	o	o	o	++	++	+ occ. necrosis
17	65	50	+	+	+	+++	++	o	o	o	o
18	85	o	++	+	o	+	o	o	o	+	+
19	50	30	++	++	o	++	++	o	o	o	o
20	65	25	++	++	o	+	o	o	+	++	+++
21	50	10	o	+	+++	o	o	+++	o	+++	++
22	85	60	++	+	o	o	o	o	++	+	+
23	80	15	o	o	++	+	o	o	+++	+	+
24	75	20	++	+	+	+	+	o	+	+	+
25	80	70	+++	++	o	+	+	o	+	+	+

Key: Membranous transf. = Membranous transformation. Occ. necrosis = Occasional necrosis.
+, ++, +++ = Degree of change; +, minimum; +++, maximum.

dividual lobules. In some instances, the lobules, on the contrary, were disorganized and fused. In general, the obsolete glomeruli no longer exhibited a lobular architecture.

In thin sections the obsolete and obsolescent glomeruli were found to have undergone changes similar in nature to those of subacute glomerulonephritis. It had been shown previously that in the subacute stage the glomeruli exhibited alterations in the intercapillary space, capillary wall and Bowman's capsule. Similar though more advanced lesions were present in the chronic stage.

Cases with involvement predominantly of the intercapillary space showed distinct scars in the lobular centers.[6] These varied in size and consisted of tortuous, rather thick fibers which stained with the periodic acid-Schiff (PAS) reagent, periodic acid-silver methenamine, and with the Mallory trichrome (chromotrope-aniline blue) stain. A small number of mononuclear cells could be found among the fibers (Figs. 1 and 2). In addition, many glomeruli contained deposits of hyalin within the intercapillary space; these stained pink with hematoxylin and eosin and red with PAS stains (Figs. 3 and 4). In the earlier stages of the disease, one could often distinguish a central capillary within, and surrounded by, the intercapillary fibers and hyalin (Fig. 5). This capillary is a normal constituent of the lobule, but is best seen in the early stage of chronic glomerulonephritis when it is clearly separated from the peripheral capillaries by fibers. As the disease progressed, the capillaries collapsed so that in the obsolete glomeruli neither the central nor the peripheral capillaries were visible. What remained was merely a fibrous stalk covered by basement membrane.

Changes of the capillary wall were most commonly caused by splitting into two layers with the creation of pericapillary spaces, or by so-called membranous transformation. The latter lesion consisted of thickening of the wall and periodic, PAS-positive cross-striation or scalloping.[7,8] When hyalin was present in the central lobular scars, it was also often found between the split layers of the capillary wall, giving rise to wire-loop-like lesions. In some cases the only significant finding was diffuse homogeneous thickening of the capillary wall. The capillary alterations were very characteristic because they persisted even in the completely collapsed glomeruli and could be identified by means of the thin section technique (Figs. 6 to 8).

Bowman's capsule often bore fibrous crescents which arose from epithelial crescents of the subacute stage (Fig. 9). More frequently there was diffuse and very striking thickening of the capsule as a result of repeated splitting and fibrosis. The thickened capsule formed a complete or nearly complete circle around the collapsed capillaries (Fig. 6).

With progression of glomerular obsolescence, the capillaries collapsed and the lumens disappeared. Often the lumen was not completely obliterated but was filled with a pale hyaline substance, probably inspissated plasma protein.

In addition to the typical features of chronic glomerulonephritis, there were obsolete glomeruli which showed merely capillary collapse without alteration of the capillary wall or the intercapillary space. These glomeruli constituted only a small percentage of the total number, and were characteristically seen in cases with considerable vascular disease and narrowing of the arteries and arterioles. They were quite similar to the glomeruli of nephrosclerosis (Figs. 10 and 11). In some, a combination of degenerative and arteriosclerotic changes was evident, i.e., thickening and splitting of the capillary wall accompanied by wrinkling of the split layers.

It is well known that many glomeruli in glomerulonephritis undergo dissolution and eventually disappear.[8] This began with gradual absorption of cells and fibers in the central lobular scars. Many small capillaries appeared around the collapsed glomerulus. Bowman's capsule lost its staining quality and eventually disappeared completely. For a while the glomerulus consisted of collapsed capillary walls (Figs. 12 and 13). Later the capillary basement membrane also disappeared.

Acute exacerbation of chronic glomerulonephritis was a fairly frequent occurrence. In 3 of our cases there was a leukocytic exudate in the glomerular capillaries and in the tubules.

The changes in the tubules consisted mainly of marked thickening and splitting of the basement membrane and atrophy of the epithelium. Eventually the tubular basement membrane underwent resorption and disappeared in a manner analogous to that in Bowman's capsule. A certain number of the tubules remained well preserved and some were dilated and hypertrophied. The intertubular stroma showed severe chronic inflammation and an increasing degree of fibrosis.

A great variation in the degree of arterio- and arteriolosclerosis was encountered (Table II). The arteriolar lesions were characterized by hyalinization and hyperplasia of the media and thickening and fibrosis of the intima. In 5 cases there was, in addition, focal necrosis of the arteriolar wall, particularly at the glomerular hilum. These lesions were similar to those described by Horn, Klemperer and Steinberg.[9]

Clinicopathologic Correlation

An attempt was made to correlate the anatomic features with some of the clinical data (Tables I and II). As a rule, the patients in the younger age group (second and third decades, cases 8, 11, 12, 17 and 19) showed

large amounts of hyalin in the glomeruli and comparatively little atrophy of the renal mass. Apparently the hyaline deposits interfere with the glomerular function and lead to earlier renal insufficiency. Older patients (over 40; cases 4, 9, 13 and 16) showed greater atrophy of kidney parenchyma, less hyaline deposit and more arteriosclerosis.

Seven patients (cases 2, 5, 6, 7, 18, 21 and 23) had exhibited the nephrotic syndrome. Two of these (cases 5 and 21) showed the typical lesions of "membranous transformation," and were 53 and 57 years old, respectively; this confirmed Bell's [1] and our [2] observations that patients with this type of lesion were usually above middle age. The other 5 patients with the nephrotic syndrome were in the youngest group of our series, with an average age of 11 years. They presented no special anatomic or histologic features.

In general, the number of obsolete glomeruli increased with the duration of disease. As observed by Allen,[10] cases with the largest number of obsolete glomeruli showed the least amount of albuminuria.

All but one of the patients in this series died in uremia, the usual cause of death in chronic glomerulonephritis. Bell's [1] series of chronic glomerulonephritis comprised 273 cases, 267 of which were described as having azotemia. This series also included 54 patients with a history of acute glomerulonephritis. In only 2 of our patients (cases 3 and 17) could such a history be elicited.

Comment

The essential feature of glomerular obsolescence is capillary collapse with loss of circulation. This is brought about by a variety of factors. McManus [4] attributed the collapse to: (a) scarring and fibrosis of the intercapillary space with encroachment upon the lumen of the capillaries; (b) crescent formation; and (c) thickening of the capillary walls. Jones [6] considered the formation of central lobular scars leading to simplification and distortion of the glomerular lobules and eventual compression of the capillaries as the important factors of obsolescence. Our observations concur with these views. The effect of the scar upon the centrilobular capillary is of special significance because the latter is completely surrounded and eventually compressed by fibrous tissue with resulting loss of circulation in the entire lobule.

Thickening of the capillary wall may interfere with glomerular filtration and lead to the capillary collapse. Though the mechanism of this "atrophy of disuse" is not clear, it undoubtedly occurs frequently. Cases with membranous transformation of the wall represent examples of pure collapse probably due to loss of function, because they show neither lobular scarring nor arteriolosclerosis.

It is conceivable that glomerular crescents, either epithelial or fibrous, exert mechanical pressure upon the capillaries. However, a more frequent feature of chronic glomerulonephritis is marked thickening of Bowman's capsule around the collapsed capillaries; this may very well represent a secondary phenomenon rather than a primary cause of capillary collapse. In those patients who develop considerable vascular sclerosis secondary to long-standing glomerulonephritis, narrowing of the arterioles contributes to glomerular obsolescence and, in some glomeruli, may be the sole cause of it.

It has been emphasized that patients with chronic glomerulonephritis rarely have a history of acute glomerulonephritis. Among Bell's cases, fewer than 20 per cent had such a history,[1] and in the present series, there were less than 10 per cent. The reverse is true in subacute glomerulonephritis. In Bell's 34 cases, the subacute disease was preceded by an acute episode in all instances. In our series of 28 cases, 6 had an unequivocal history of an acute episode, and in another 10 there was a probable antecendent. Since the glomerular lesions of the chronic stage are basically identical with those of the subacute stage, it must be assumed that the patients with the chronic lesions had forgotten the acute episode. It is less likely that the acute episode was very mild or that there are two types of chronic glomerulonephritis, one secondary to an acute attack and the other primary and insidious from the start. These two forms are morphologically indistinguishable. The only apparent exceptions are the cases of pure membranous transformation which exhibit very little evidence of inflammation and possibly arise by an entirely different mechanism. Clinically, these patients fall into Class 2 of Ellis' classification.[11]

As a rule, it is possible to distinguish the contracted kidney of chronic glomerulonephritis from that of nephrosclerosis and of pyelonephritis. This distinction is based upon the gross appearance of the kidney, the type of tubular alterations, and the structure of the partly preserved glomeruli. However, in some instances this is not readily accomplished even when both kidneys are available for examination. The problem becomes much more difficult when diagnosis is attempted on a small fragment of kidney tissue obtained by needle biopsy, particularly when all the glomeruli are obsolete or obsolescent. In such a case, the presence of typical changes in the intercapillary spaces and particularly in capillary walls may be very helpful.

Summary and Conclusions

Twenty-five examples of chronic glomerulonephritis were studied by means of thin sections. Lesions observed in obsolete and obsolescent

glomeruli correspond to those seen in subacute glomerulonephritis and consisted of intercapillary fibrosis and alterations in capillary walls and Bowman's capsule. These were accompanied by capillary collapse and eventual absorption and disappearance of the glomeruli.

The causes of capillary collapse are found among the following: (a) intercapillary fibrosis; (b) thickening of the capillary walls; (c) crescent formation; and (d) sclerosis of afferent arterioles. Intercapillary fibrosis leads to constriction of centrilobular capillaries, as well as compression of peripheral capillaries.

The number of obsolete glomeruli was roughly in proportion to the duration of the disorder. Cases with extensive hyaline deposits in the glomeruli showed more rapid progression of the disease and an earlier onset of uremia.

The presence of typical lesions in the intercapillary spaces and in capillary walls may be helpful in diagnosis of chronic glomerulonephritis, particularly on renal biopsy specimens.

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[*Illustrations follow*]

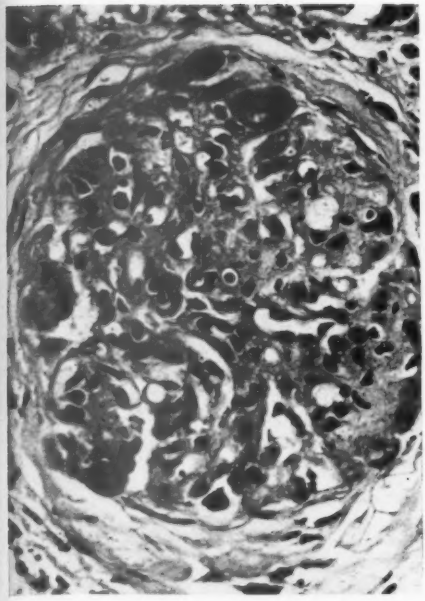
Legends for Figures

Fig. 1. Case 4. Obsolescent glomerulus. There is fibrosis of lobules. A number of mononuclear cells are apparent. Minimal hyaline deposit (dark nodules) is seen at the top and the left lower edge of the glomerulus. Standard section; hematoxylin and eosin stain. × 385.

Fig. 2. Case 4. Lobule of an obsolescent glomerulus. Fibers and mononuclear cells appear between compressed capillaries. Thin section; periodic acid-Schiff (PAS) stain. × 1500.

Fig. 3. Case 3. An obsolete glomerulus showing extensive hyalinization. Standard section; hematoxylin and eosin stain. × 385.

Fig. 4. Case 3. A lobule of an obsolete glomerulus showing hyaline deposit in the center and splitting of the walls of peripheral capillaries. Thin section; PAS stain. × 1500.



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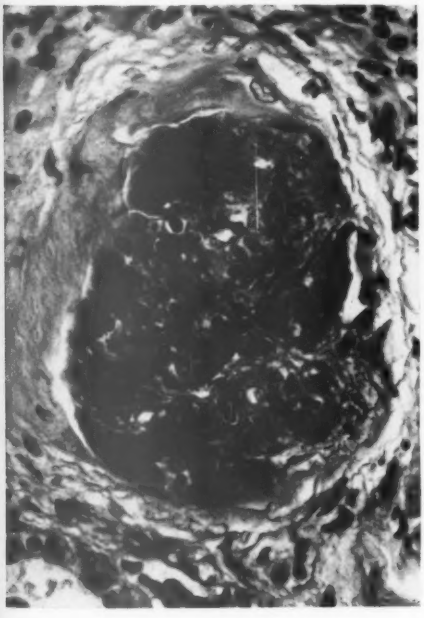

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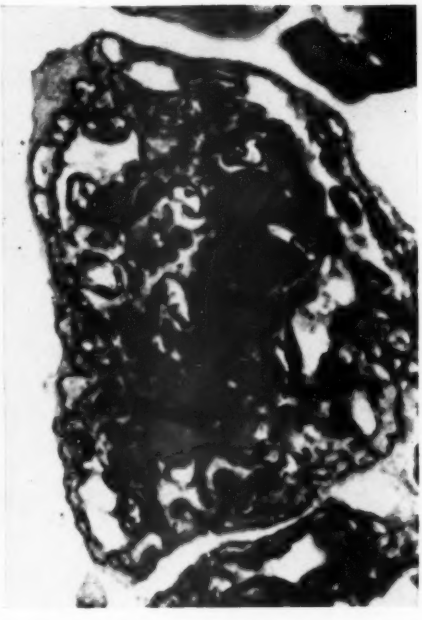

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FIG. 5. Case 4. A lobule of an obsolescent glomerulus, showing a central lobular capillary surrounded by fibers and opening into a peripheral capillary. Thin section; PAS stain. × 1500.

FIG. 6. Case 21. Obsolete glomerulus. Standard section; hematoxylin and eosin stain. × 385.

FIG. 7. Case 21. A portion of an obsolete glomerulus with collapsed capillaries. The characteristic changes of membranous transformation ("cross striation") are visible in some of the capillary walls (arrow). Thin section; PAS stain. × 1500.

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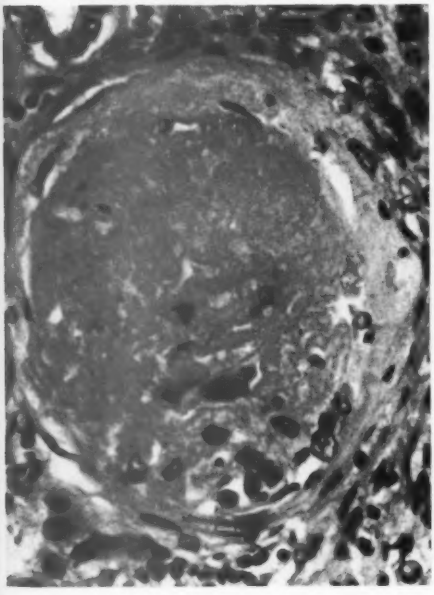

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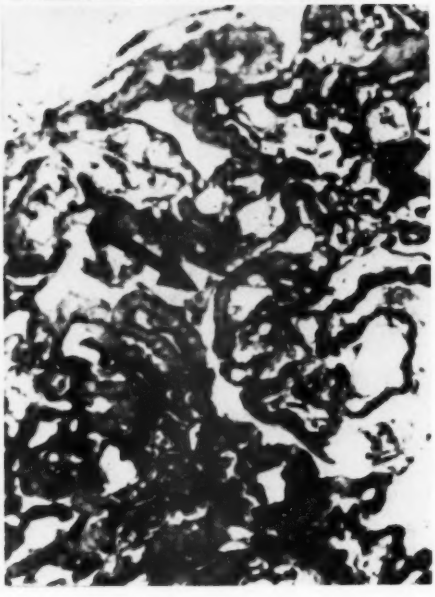

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FIG. 8. Case 19. Obsolete glomerulus. Hyaline deposits are evident in some of the capillary walls ("wire loops") (arrows). Thin section; PAS stain. × 1500.

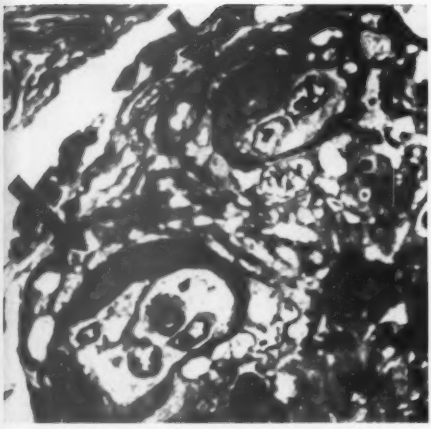
FIG. 9. Case 16. Collapsed capillaries (dark staining) are surrounded by a large fibrous cresent. Thin section; PAS stain. × 1500.

FIG. 10. Case 9. Obsolete glomerulus. Standard section; hematoxylin and eosin stain. × 385.

FIG. 11. Case 9. A portion of an obsolete glomerulus with collapsed capillaries. Normal capillary walls. Thin section; PAS stain. × 1500.

FIG. 12. Case 1. A glomerulus in the process of resorption. Note the loss of Bowman's capsule and the presence of dilated capillaries along the periphery. A glomerular arteriole is seen in the right lower corner. Standard section; hematoxylin and eosin stain. × 385.

FIG. 13. Case 1. Resorption of a glomerulus. There are collapsed capillaries and a few cells. Bowman's capsule is absent. Dilated periglomerular capillaries are seen along the upper border. Thin section; PAS stain. × 1500.



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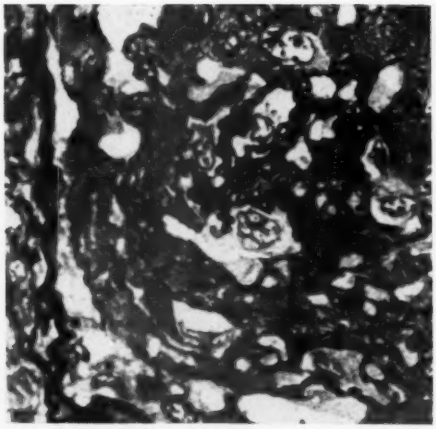

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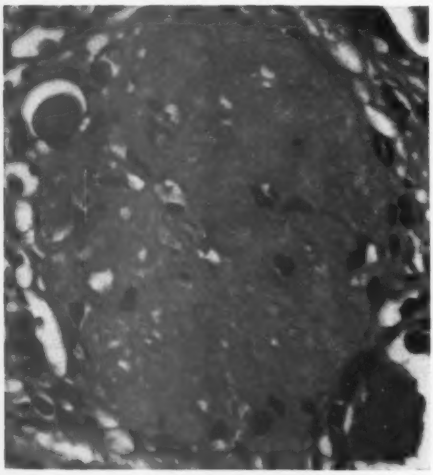

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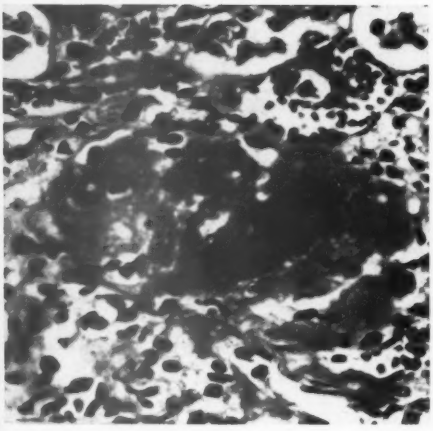

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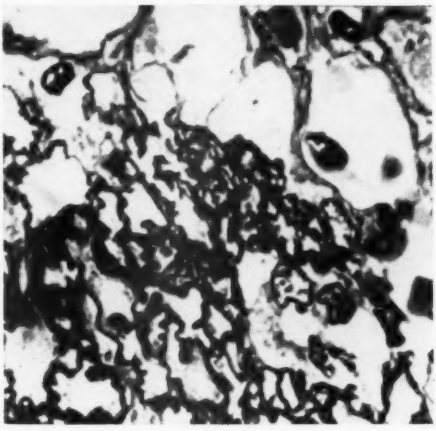

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